

Deletion of polyphosphate kinase gene (*ppk*) has a stimulatory effect on actinorhodin production by *Streptomyces coelicolor* A3(2)

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Abstract: Polyphosphate, which is synthesized by the enzyme polyP kinase (PPK), is an important energy and phosphate storage polymer that plays a crucial role in the regulation of adaptive responses of cells to physical and chemical stresses. In this work, the polyphosphate kinase gene (*ppk*) of *Streptomyces coelicolor* was deleted, and the effect of this mutation on actinorhodin and undecylprodigiosin biosynthesis was investigated. Deletion of the *ppk* gene had a stimulatory effect on actinorhodin production; the mutant strain produced about 5 times more antibiotic compared to the wild type strain at 120 h of growth. There was no difference in undecylprodigiosin production between the mutant and wild-type strains. In the presence of a selective antibiotic, the mutant strain could grow only on rich medium and could not sporulate effectively. Moreover, while the wild type strain was resistant, the mutant strain was sensitive to H₂O₂ in the conditions tested. Mutant characters were complemented by the *ppk* gene, which is cloned on a high copy number plasmid.

Key words: *Streptomyces*, polyP, polyphosphate kinase, *ppk*, antibiotic production

Introduction

Polyphosphate (polyP) is found in the cells of every archeon, bacterium, fungus, plant, insect, and mammal. It is a linear polymer of phosphate molecules joined together by high-energy anhydride bonds (1). This polymer is synthesized by polyP kinase (PPK) using the terminal phosphate of ATP as the substrate (2) and degraded to inorganic phosphate by endopolyphosphatase (PPN) and exopolyphosphatase (PPX) enzymes (3). PolyP has been associated with energy producing cellular structures, membranes, and nucleic acids (4) and can be used as an ATP substitute by some kinases (5). This polymer serves as a phosphate reservoir that is mobilized in conditions of Pi starvation (6), and it is an important energy store (5). PolyP is required for the stationary-phase survival of bacteria and plays an important role in the regulation of adaptive responses to physical and chemical stresses (7). It

also aids in maintaining optimal transcription (8) and translation (9) efficiency. There are also some studies showing polyP use as a potential alternative method for addressing heavy metal and phosphate contamination by bioremediation. It has also been shown that polyP is necessary for motility, biofilm formation, and other virulence properties in pathogenic bacteria (10-12).

Streptomyces are soil-dwelling bacteria that synthesize a wide variety of bioactive compounds, including 75% of commercial antibiotics. In *Streptomyces*, the biosynthesis of antibiotics is controlled by a carbon and nitrogen source and inorganic phosphate. Several antibiotics were known to be produced only under phosphate-limiting nutritional conditions, and antibiotic biosynthesis was repressed in high phosphate concentrations in *Streptomyces* (13). To our knowledge, there are few studies about the relation of polyP, which is an

important phosphate reservoir inside the cell, with antibiotic biosynthesis in *Streptomyces*. Chouayekh and Virolle (14) constructed a disruptive mutant of *S. lividans* TK24 *ppk* gene, and they reported that the interruption of the *ppk* gene led to an increase in antibiotic biosynthesis in *S. lividans*. In a different study, the same group proposed that this increase in antibiotic biosynthesis in a disruptive mutant of *ppk* is triggered by the energetic balance of the cell (15). In our study, the *ppk* gene of *Streptomyces coelicolor* was deleted by a PCR-mediated method, and the effect of this mutation on actinorhodin and undecylprodigiosin biosynthesis was quantified in liquid R2YE cultures. Moreover, oxidative stress sensitivity levels of the mutant and wild type strains were compared.

Materials and methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in the Table. *E. coli* strains were grown in LB liquid and solid medium at 30 °C or 37 °C. The *E. coli* ET12567 (16) containing RP4 derivative pUZ8002 (17) was used as the nonmethylating plasmid donor for intergeneric conjugation with *S. coelicolor* A3(2) (18). *E. coli* BW25113 (19) was used to propagate the recombination plasmid pIJ790 and *S. coelicolor* cosmid Std84. *S. coelicolor* strains were grown in TSB, YEME, and R2YE medium at 30 °C. Ampicillin (100 µg/mL), apramycin (50 µg/mL), chloramphenicol (25

µg/mL), and kanamycin (50 µg/mL) were added to growth media as required.

DNA Procedures

Isolation of plasmids and cosmids, restriction enzyme digestions, and agarose gel electrophoresis were performed according to standard molecular biology techniques (20). *E. coli* strains were transformed with plasmids and cosmids by standard chemical method (20). Conjugation and transformation conditions for *Streptomyces* species were performed as described by Kieser et al. (21). Isolation of *Streptomyces* total DNA was performed as previously described (21). Southern blot analysis was performed as described by Sambrook et al. (20), and DNA fragments used as probes were labeled with digoxigenin using a random priming kit (DIG DNA labeling mix; Roche Biochemicals, Basel, Switzerland).

Deletion of *ppk* gene of *S. coelicolor* A3(2)

Deletion of the *ppk* gene of *S. coelicolor* A3(2) was performed using a PCR-based system (Figure 1) (22). The gene disruption cassette *aac*(3)IV and the oriT were amplified from pIJ773 using the primers F:5’aaccatcgat tcagccagccttgcgaggctcaccgaatgATTCCGGGGATCCGT CGACC3’ and R:5’gtttccgggtggaaagtcctgcggaatgtccgtcgg gtcaTGT AGGCTGGAGCTGCTTC3’ [F and R primers have 39 identical nucleotides with the upstream and downstream region of the *ppk* gene of *S. coelicolor* DNA, respectively (lowercase in both primers) and 20 or 19 identical nucleotides with pIJ773 DNA, respectively

Table. Bacterial strains, cosmids, and plasmids.

Bacterial strains	Relevant genotype/comments	References
<i>E. coli</i> BW25113	<i>K12 derivative:ΔaraBAD, ΔrhaBAD</i>	(19)
<i>E. coli</i> ET12567	<i>dam, dcm, hsdS, cat, tet</i>	(16)
<i>S. coelicolor</i> A3(2)	Prototrophic wild type	(18)
<i>S. coelicolor</i> Δ <i>ppk</i>	<i>ppk</i> deletion mutant strain	this study
Cosmids and plasmids		
Std84	Cosmid containing the <i>ppk</i> gene	Sanger institute
pHZ1351	<i>tsr Ltz– sti+</i>	(21)
pIJ773	<i>aac</i> (3)IV	(22)
pIJ790	l-RED (<i>gam, bet, exo</i>), <i>cat, araC, rep101^{ts}</i>	(22)
pUZ8002	<i>tra, neo</i> , RP4	(17)
pBluescript II SK	<i>E. coli</i> vector Ap ^R <i>lacZ</i> arif1	Stratagene
pSK <i>ppk</i>	<i>ppk</i> gene cloned into pBluescript II SK	this study
pHZ <i>ppk</i>	<i>ppk</i> gene cloned into pHZ1351	this study

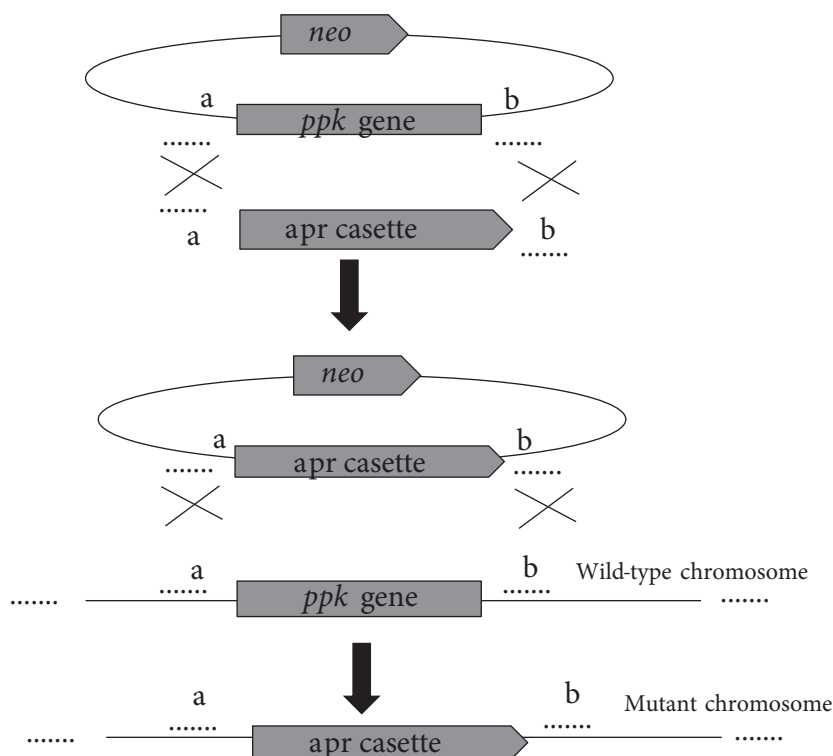


Figure 1. Strategy used for the generation of *Streptomyces coelicolor* *ppk* mutant. Dotted lines a and b indicate the 39 nucleotides of the primers that are identical to the regions flanking the *S. coelicolor* *ppk* DNA sequence. The apramycin cassette with oriT region was amplified from pIJ773 and introduced into the *E. coli* BW25113/pIJ790 containing the Std84 cosmid, which contained the *ppk* gene sequence. Recombinant cosmids were transferred to *S. coelicolor* by protoplast transformation, and double-crossover mutants were screened by apramycin resistance and kanamycin sensitivity.

(uppercase)]. The disruption cassette was amplified by PCR, and *E. coli* BW25113/pIJ790 bearing cosmid Std84 was transformed with this cassette. Isolated mutant cosmids were verified by restriction analysis and PCR and then introduced into nonmethylating *E. coli* ET12567 containing the RP4 derivative pUZ8002. Then the mutant cosmid was transferred to *S. coelicolor* by protoplast transformation (23) since no exconjugants were obtained by intergeneric conjugation after several trials (22). One of the Apr^R and Kn^R transformants, which is presumed to contain the entire cosmid integrated into the chromosome by a single crossover event, was passed through 5 rounds of non-selective cultivation in R2YE to facilitate the second crossover. Then recombinants were screened for their kanamycin sensitivity and apramycin resistance to identify double crossover transformants.

Actinorhodin and undecylprodigiosin determination

Actinorhodin and undecylprodigiosin production were determined spectrophotometrically as

described by Kieser et al. (21). To determine the total actinorhodin and lactone form of the antibiotic γ -actinorhodin, 1 mL of culture was treated with 250 μ L of KOH (5 M) at 4 °C overnight and centrifuged (13,000 rpm for 5 min). Then the A_{640} of the supernatant was determined. Molarity was quantified by the formula:

$$\text{absorbance} = \epsilon \times [M],$$

where $\epsilon_{640} = 25,320$ for the pure compound.

To determine the undecylprodigiosin (a mixture of at least 4 prodiginines), the mycelium (pellet from actinorhodin measurement) was washed 2 times with 0.5 M HCl. Then the pellet was dissolved in 1 mL of a methanol-HCl (0.5 M) mixture and incubated at room temperature for 2 h. Then the A_{530} of the supernatant was determined ($\epsilon_{530} = 100,500$).

Complementation of *S. coelicolor* Δppk mutant

A 2890-bp DNA fragment containing the *ppk* coding region was amplified using F: 5'AACTGCA

GAACCAATGCATTGGCGCGCAGGAACTCGG TG 3' (*Pst*I cutting site is bold and underlined) and R: 5'**CGGGATCCCCGCGTACGGCGAGGA** GGGGTAC 3' (*Bam*HI cutting site is bold and underlined) primers and cloned into the *Bam*HI and *Pst*I digested pBluescript SK plasmid giving rise to pSK*ppk*. Then a 3015 bp *Xba*I+*Hind*III fragment of pSK*ppk* containing the *ppk* gene was cloned into the *Xba*I+*Hind*III site of pHZ1351(8300 bp). The new construct (pHZ*ppk*, 11,315 bp) isolated from *E. coli* ET12567 was used to transform *S. coelicolor* Δ *ppk* protoplasts. One of the thiostrepton-resistant transformants was selected and analyzed for its antibiotic production and viability on MS, TSA, and TBO media.

Oxidative stress

Vegetative cells of *S. coelicolor* wild type (10^8) and Δ *ppk* strains were plated on rich medium R2YE to which 0.37 mM, 1 mM, 1.85 mM, and 3.7 mM KH_2PO_4 was added. After spreading, a Whatman paper disk wetted with 10 μL of 100 mM H_2O_2 was placed in the center of the plate. After 3 days of incubation at

30 °C, sensitivity and/or resistance of the strains to oxidative stress were determined by measuring the diameters of the zones of growth inhibition.

Results

Deletion of the *ppk* gene of *S. coelicolor* A3(2)

Red/ET recombination methodology (22) was used to delete the *S. coelicolor* *ppk* gene. According to this method, the mutant cosmid, in which the *ppk* gene was replaced by the Apr^RoriT cassette, is transferred to *S. coelicolor* by conjugation. However, our attempts to obtain exconjugants failed, and the mutant cosmid was transferred to *S. coelicolor* by protoplast transformation (see experimental procedures). Among *S. coelicolor* transformants, 1 apramycin-resistant and kanamycin-sensitive transformant was isolated, and Δ *ppk* mutation was verified by Southern hybridization of *Xho*I-digested chromosomal DNA. A hybridization band of 1815 bp was obtained only for the wild-type strain using a *ppk* fragment (1815 bp) as probe (Figure 2). Although the Δ *ppk* mutant

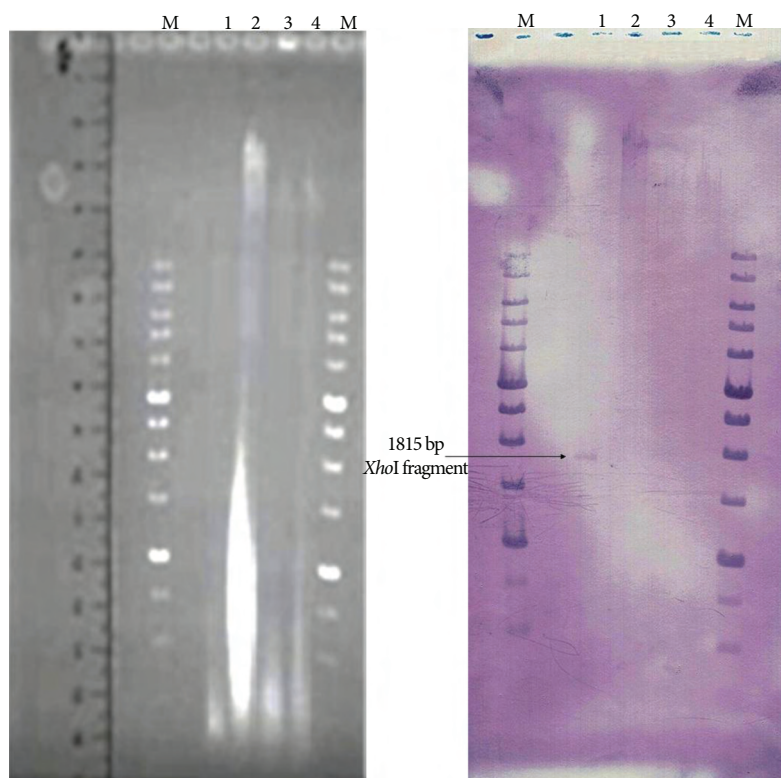


Figure 2. Verification of *ppk* deletion by Southern blot hybridization; (a) agarose gel showing the pattern of the *Xho*I-digested chromosomal DNA of the wild-type *S. coelicolor* (lane 1) and the mutant (lanes 2-4), (b) hybridization pattern of the *Xho*I-digested chromosomal DNA of the wild-type *S. coelicolor* (lane 1) and the mutant (lanes 2-4) probed with the *ppk* fragment. Size marker is 1 kb DNA marker (Bioron).

strain grew on rich selective medium R2YE, it did not grow on some of the selective media (MS, TSA, and TBO; data not shown). These results explain why exconjugants were not obtained since MS medium was used in the screening procedure.

S. coelicolor Δppk strain produces more actinorhodin than the wild type strain

Actinorhodin and undecylprodigiosin production of *S. coelicolor* wild type and Δppk strains were measured in liquid R2YE cultures for 168 h. Growth of wild type and mutant strains were nearly identical in this medium (Figure 3a). The Δppk strain produced about 5 times more actinorhodin than the wild type at 120 h of growth (Figure 3b). However, no significant increase in undecylprodigiosin production was found in the mutant strain compared to the parental strain (Figure 3c). These results were consistent in repeated experiments.

Enhanced production of actinorhodin by the mutant strain was also seen on solid R2YE medium prepared with different KH_2PO_4 concentrations (Figure 4). Increased production of the antibiotic was diminished by the addition of 1.48 mM KH_2PO_4 to the R2YE (Figure 4); however, antibiotic production in the mutant strain was higher than in the wild type strain.

Complementation of the *S. coelicolor* Δppk mutation

When pHZ*ppk* plasmid, which carries the *ppk* gene, was transferred into *S. coelicolor* Δppk strain the actinorhodin overproduction phenotype of the mutant strain on solid R2YE was abolished (Figure 5), demonstrating that the mutant phenotype resulted from the deletion of the *ppk* gene. After complementation with pHZ*ppk* plasmid, the mutant strain gained the ability to grow on selective MS, TSA, and TBO media (data not shown).

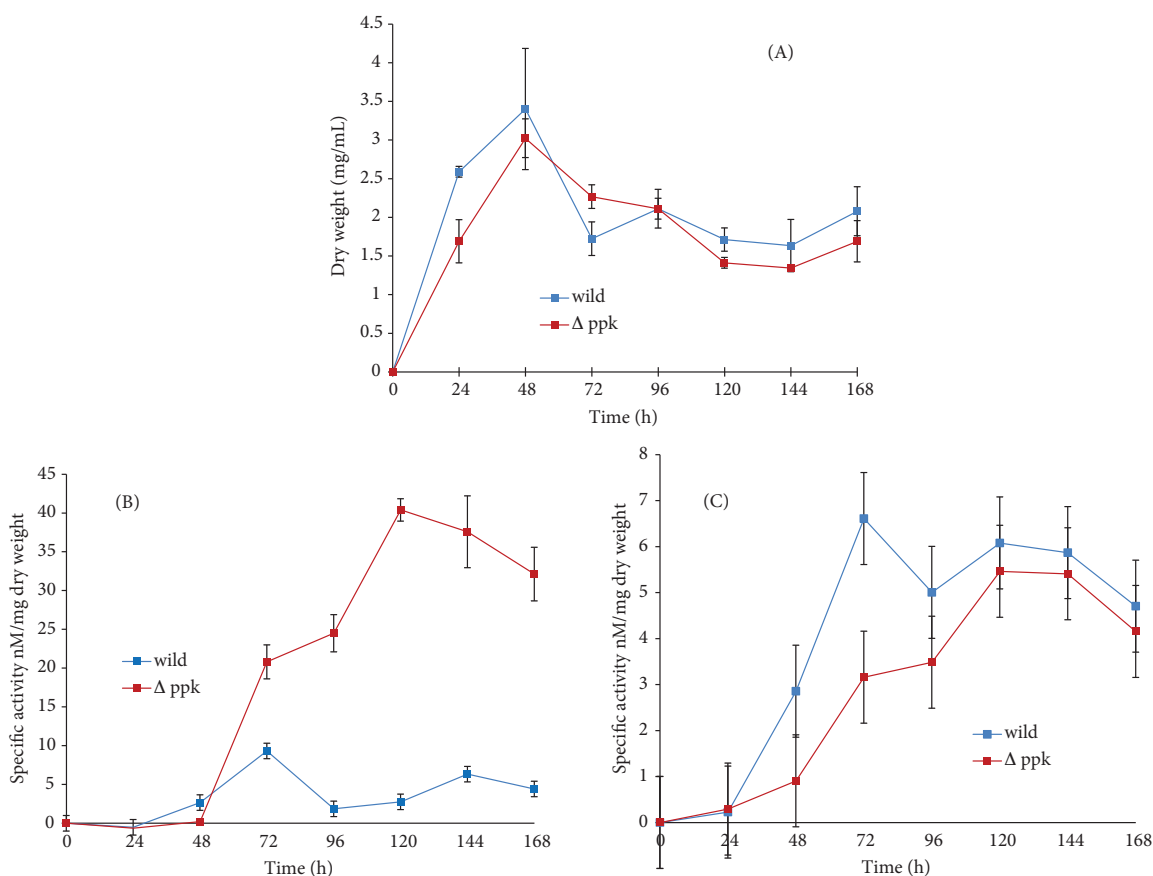


Figure 3. Growth (mg dry weight/mL) (A) and specific production (nmol/mg dry weight) of actinorhodin (B) and undecylprodigiosin (C) by *S. coelicolor* A3(2) (wild) and *S. coelicolor* Δppk (Δppk). Liquid cultures of wild and mutant strains were produced in R2YE medium. Vertical bars indicate standard deviation from the mean value. Note the high levels of actinorhodin produced by Δppk (B).

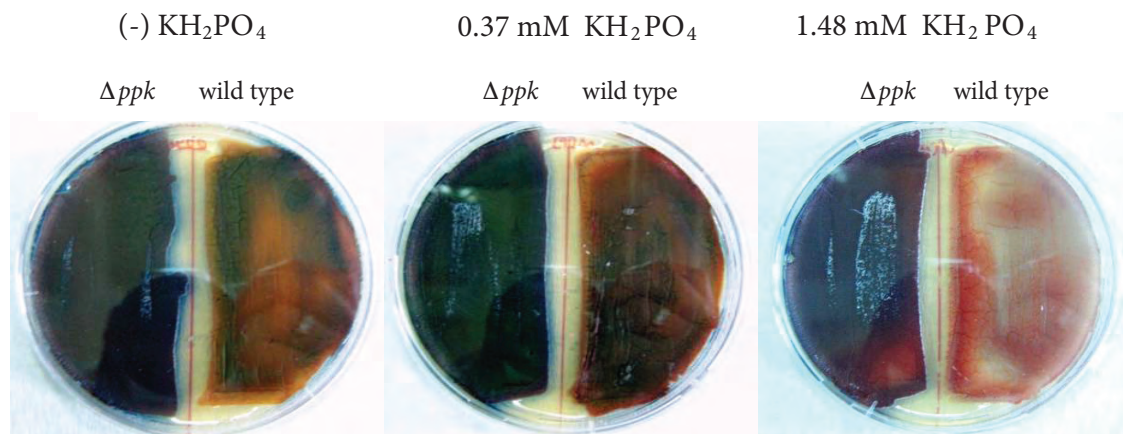


Figure 4. Production of antibiotic by *S. coelicolor* A3(2) and *S. coelicolor* Δppk strain on solid R2YE medium with no KH_2PO_4 , 0.37 mM KH_2PO_4 , and 1.48 mM KH_2PO_4 added. Plates were incubated for 4 days at 30 °C.

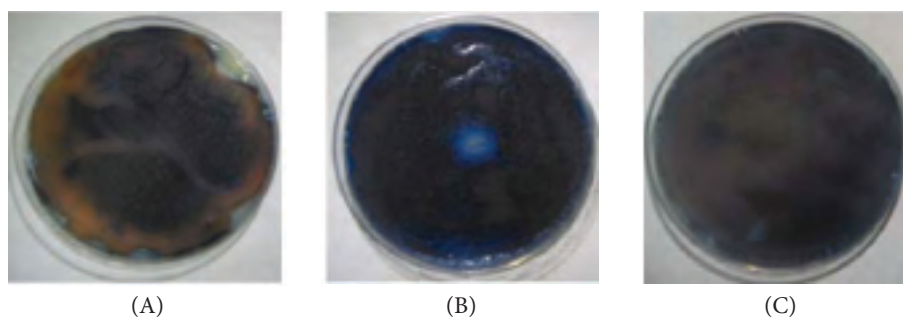


Figure 5. Antibiotic production in *S. coelicolor* A3(2) parental (A) and *S. coelicolor* Δppk mutant strain before (B) and after (C) complementation with pHZ*ppk*.

H_2O_2 sensitivity of *S. coelicolor* A3(2) and *S. coelicolor* Δppk strains

The sensitivities of wild type and *ppk* mutant strains to H_2O_2 were compared on solid R2YE medium containing different phosphate concentrations. Diameters of the zones of growth inhibition showed that, although the *ppk* mutant strain was sensitive to 10 μL H_2O_2 at 100 mM concentration, the wild type strain was resistant to the same concentration of H_2O_2 (Figure 6). Results were not affected by different phosphate concentrations in the medium.

Discussion

A linear polymer consisting of tens to hundreds of phosphate molecules, polyP performs important roles in all living cells. There are very few studies that relate polyP metabolism with secondary metabolite production. In this study, the *ppk* gene of *Streptomyces coelicolor* A3(2) was deleted, and the effect of this mutation on antibiotic biosynthesis was determined.

Deletion of the *ppk* gene had a stimulatory effect on actinorhodin production; however, it did not affect undecylprodigiosin production. Enhanced production of actinorhodin by the mutant strain was also seen on solid R2YE medium.

Strong actinorhodin production was also observed upon disruption of the *ppk* gene of *S. lividans* TK24 (14,15). This increased production of antibiotic correlated with enhanced transcription of the specific activator gene (*actII-ORF4*) of the actinorhodin pathway. In the same study, transcription of the *redD* encoding the specific activator of the undecylprodigiosin biosynthetic pathway increased to a lesser extent. Consistent with these results, in our study no significant change in undecylprodigiosin production by the mutant strain was found compared to the parental strain.

Antibiotic biosynthesis in streptomycetes is triggered by nutritional limitation in inorganic phosphate (Pi) (13,24-26), which, in turn, may be

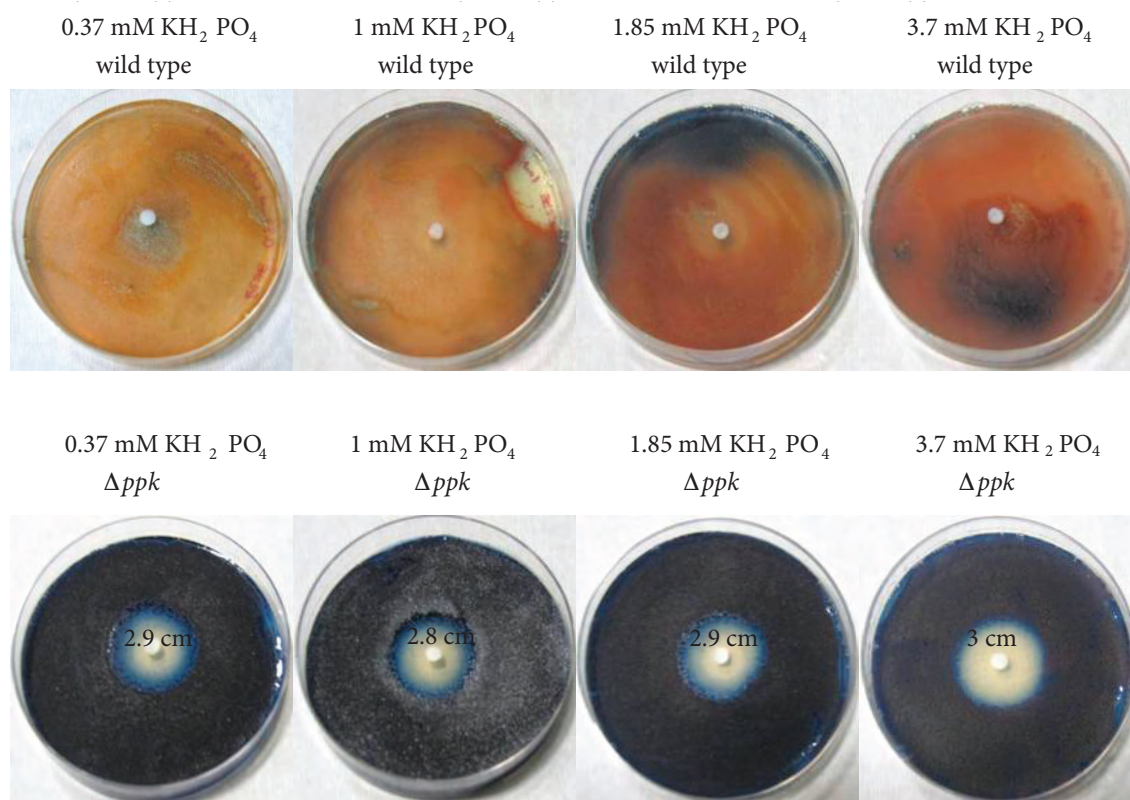


Figure 6. H_2O_2 sensitivity test. A total of 10^8 vegetative cells of *S. coelicolor* A3(2) and *S. coelicolor* Δppk were spread on R2YE medium to which 0.37 mM, 1 mM, 1.85 mM, and 3.7 mM KH_2PO_4 was added. Sensitivity of the strains to 10 μL of 100 mM H_2O_2 was determined after 3 days of incubation at 30 $^\circ\text{C}$ by measuring the diameters of the zones of growth inhibition.

correlated with a low energetic charge (27,28). As proposed by Ghorbel et al. (15) in *S. lividans* TK24 mutant strain with interrupted *ppk* gene, in conditions of Pi scarcity, *S. coelicolor* *ppk* deletion mutant strain, which lacks an important ATP-regenerating enzyme, could have a lower intracellular adenylate charge than the wild type strain and overproduce antibiotic.

In the present study, *S. coelicolor* *ppk* mutant strain was sensitive to H_2O_2 compared to the wild type strain under the conditions tested. The same H_2O_2 sensitivity was seen in *S. lividans* TK24 *ppk* mutant strain (15). Activation of the central metabolic pathways in Pi-limited conditions generates internal oxidative stress, which is deleterious for the cell. Since the *ppk* mutant strain is more affected by Pi scarcity than the wild type strain, highly activated ATP-producing central metabolic pathways could cause higher oxidative stress in the mutant strain than the wild type strain (15).

There are only a few studies that investigate the role of polyP in *Streptomyces*. In this study we have

shown the negative effect of the *ppk* gene (encoding the enzyme responsible for polyP synthesis/degradation) on antibiotic production by *S. coelicolor*. Our results support the idea proposed by Chouayekh and Virolle (14) that it is possible to engineer the cycle of synthesis/degradation of the polyP to control antibiotic biosynthesis in *Streptomyces* species.

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