Toward A Better Understanding Of Why And How Antibiotic Biosynthesis Is Being Triggered In *Streptomces* ?

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Streptomyces are Gram + filamentous bacteria, of great industrial interest. They already produce the majority of the antibiotics used in modern medicine but the sequencing of the genome of some members of this genera revealed that they have the genetic potentiality to produce much more. In *Streptomyces*, antibiotic production usually occurs in the periods of slow or no growth and is triggered by a nutritional limitation in phosphate limitation. Antibiotic production can thus be considered as resulting from an adaptation to growth in condition of phosphate limitation.

A novel understanding of the regulation of antibiotic biosynthesis was inferred from the analysis of a mutant of *Streptomyces lividans*, a naturally very weak antibiotic producing strain, that became a very high producer of some coloured antibiotics, upon the interruption of a single gene, called *ppk* (Chouayekh and Virolle, 2002). This gene was shown to encode an enzyme acting, *in vitro*, as a polyphosphate kinase (PPK), polymerising the γ phosphate of ATP into polyphosphate when the ATP/ADP ratio in the reaction was high and as a nucleoside di-phosphate kinase (NDPK), regenerating ATP from ADP and polyphosphate, when this ratio was low (Chouayekh and Virolle, 2002). *In vivo*, the expression of *ppk* was shown to be higher in condition of Pi limitation (condition correlating with a weak energetic charge) than in condition of phosphate sufficiency (Ghorbel et al., 2006a). In condition of phosphate limitation, the expression of *ppk* was shown to be positively controlled (likely indirectly) by the two components system PhoR/PhoP whereas in condition of phosphate sufficiency, the expression of *ppk* is thought to be negatively controlled by a repressor using ATP as co-repressor (Ghorbel et al., 2006a).

In order to assess the *in vivo* enzymatic function of Ppk, in condition of phosphate limitation, a comparative analysis of the intracellular content in ATP, ADP and polyphosphate of the wild type strain of *S. lividans* and of the *ppk* mutant was carried out. At the beginning of growth, polyphosphates were always found to be longer and more abundant in the *ppk* mutant strain than in the *wt* strain suggesting that, *in vivo*, Ppk might act as an ATP regenerating enzyme (Ghorbel et al., 2006a). The absence of this enzyme in the *ppk* mutant, was indeed shown to result in an higher intracellular concentration of ADP than in the wild type strain suggesting a default of ADP to ATP regeneration in that strain. Furthermore, at late incubation times, several observations suggest that a strong activation of some specific enzymes of central metabolism takes place in the *ppk* mutant strain. This strong activation, accompanied by a raise of the internal oxidative stress, results in the production of ATP, reduced cofactors (NADH) and carbon skeletons that will not be used for anabolism but will constitute "building blocks" for antibiotics biosynthesis.

Introduction

There is an urgent need to found new drugs leads to face a major threat of this century, the development of multi resistant pathogenic bacteria. Actinomycetes are already providing most of the antibiotics used in modern medicine, but the sequence of the genome of a few species of this large genera (mainly *Streptomyces* species, *S. coelicolor*, *S. avermilis, S. scabies*, *S. ambofaciens*) revealed that they possess 5 to 10 fold more biosynthetic pathways that could have been predicted from their biosynthetic abilities (each specie is known to produce 3 to 4 different antibiotics). This enormous genetic diversity is likely to result in the production of secondary metabolites with different chemical structures and bio-activities. A very important and unexploited reservoir of genetic and metabolic diversity resides in the thousand of species of this genus. However, the expression of these putative biosynthetic pathways, detected *in silico*, is often weak and the resulting metabolites too poorly produced to be characterised. A major challenge is to find ways to exploit the outstanding genetic potential and thus metabolic richness of the several hundreds of Actinomycetes species present on earth in order to discovery new active drugs. In order to conceive strategies to exploit this huge metabolic diversity, we need to get a deeper understanding of what triggers the expression of the biosynthetic pathways and what is the nature of the regulatory changes that lead the cell to redirect its central metabolism, usually aimed at biomass construction, toward the production of secondary metabolites.

Some of our recent results leads us to propose a novel view of the regulation of antibiotic biosynthesis that has high explicative and predicative value. This novel view was inferred from the analysis of a mutant of *Streptomyces lividans*, a naturally very weak antibiotic producing strain, that became a very high producer of the coloured antibiotics, actinorhodin (ACT) and undecylprodigiosin (RED) upon the interruption of a single gene, called *ppk* (Chouayekh and Virolle, 2002).

The *Ppk* Gene Of *S. Lividans* Plays A Negative Role In The Regulation Of Antibiotic Biosynthesis

In vitro enzymatic function of Ppk

The *ppk* gene was shown to encode an enzyme acting, *in vitro*, as a polyphosphate kinase (PPK), polymerising the γ phosphate of ATP into polyphosphate (long polymers of phosphate linked by high energy phosphoanhydride bonds) when the ATP/ADP ratio in the reaction mix is high and as a nucleoside di-phosphate kinase (NDPK), regenerating ATP from ADP and polyphosphates, when this ratio is low (1).

$$nATP \underset{NDPK}{\overset{PPK}{\rightleftharpoons}} PolyPn + n\ ADP$$

Regulation of *ppk* expression

The expression of *ppk* was shown to be higher in condition of phosphate limitation, condition resulting in a weak energetic charge, than in condition of phosphate sufficiency (Ghorbel et al., 2006a).

In condition of phosphate limitation, the expression of *ppk* was shown to be under the positive control (likely indirectly) of the two components system (TCS) PhoR/PhoP. In these conditions, the expression of *phoR/phoP* is auto-induced and PhoR/PhoP controls the expression of numerous genes involved in the supply of phosphate either from external (high affinity phosphate transporters, secreted phosphatases etc…) or internal sources (enzymatic systems responsible for the degradation of phosphate rich cellular components such as polyphosphate, phospholipids, nucleic acids etc…). However since this TCS also controls the expression of *ppk*, it is also obviously involved in maintaining the energetic balance of the cell (see below). All together these genes constitute the *pho* regulon.

In condition of Pi sufficiency, the expression of *ppk* is thought to be repressed by a putative repressor revealed in DNA band shift experiments (Ghorbel et al., 2006a). *In vitro*, the affinity of this repressor for its target sites in the *ppk* promoter region is enhanced in the presence of ATP, suggesting that ATP might act as a co-repressor of *ppk* expression. The expression of *ppk* might thus be directly regulated by the energetic state of the cell, repressed when the intracellular concentration of ATP is above a certain threshold (condition of phosphate sufficiency) and de-repressed/induced when this concentration falls below a certain threshold (condition of phosphate limitation).

Comparative analysis of the intracellular content in ATP, ADP and polyphosphate of the wild type strain of *S. lividans* and of the *ppk* mutant:

In order to assess the *in vivo* enzymatic function of Ppk, in condition of Pi limitation, a comparative analysis of the intracellular content in ATP, ADP and polyphosphate of the wild type strain of *S. lividans* and of the *ppk* mutant was carried out.

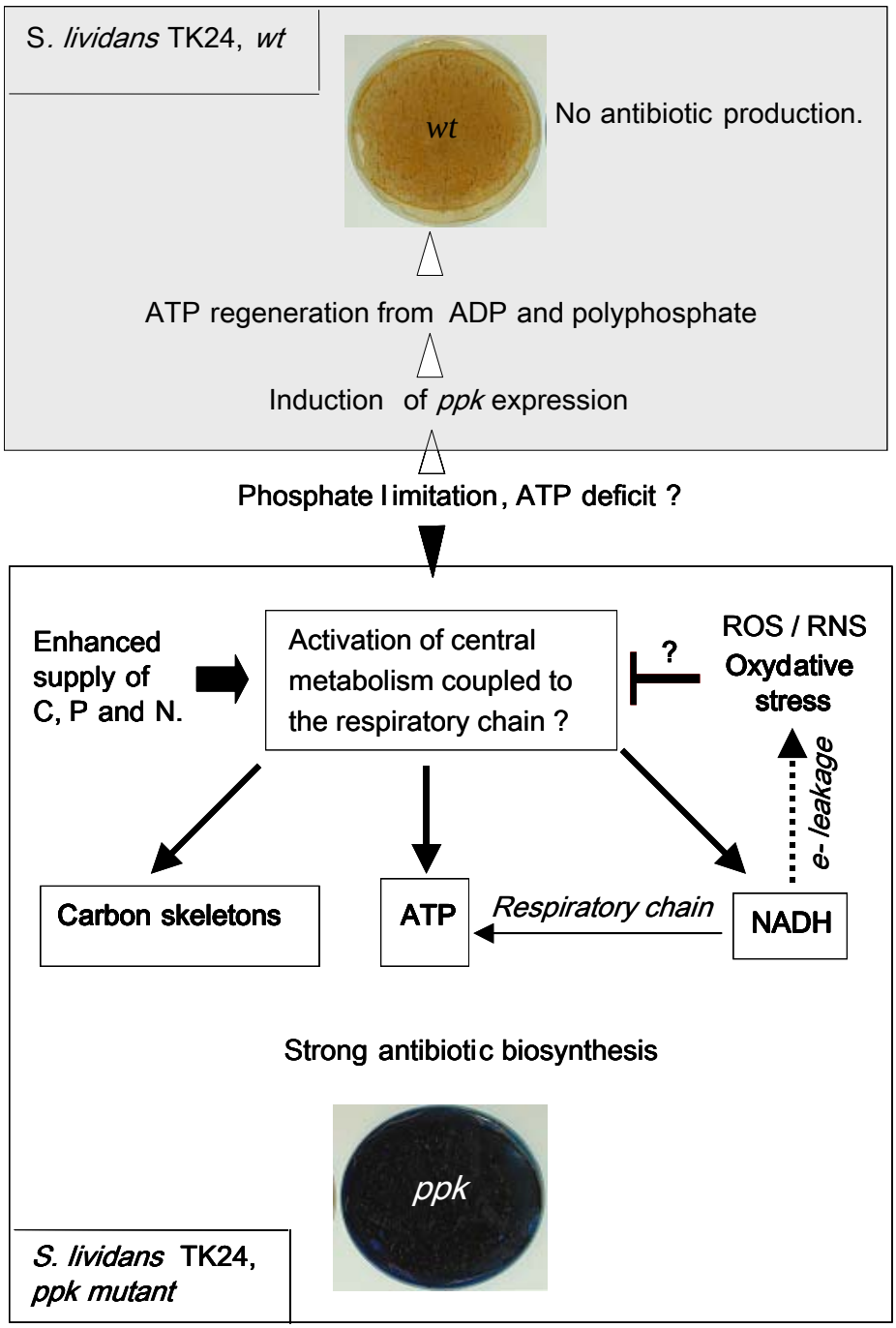
At the beginning of growth, polyphosphates (whose biosynthetic origin is unknown) were always found to be longer and more abundant in the *ppk* mutant strain than in the *wt* strain suggesting that, *in vivo*, Ppk might acts as a Nucleoside Di Phosphate Kinase (NDPK), regenerating ATP from ADP and polyphosphates. The absence of this enzyme in the *ppk* mutant, was indeed shown to result in an higher intracellular concentration of ADP than in the wild type strain, all along growth (C. Esnault, unpublished results), suggesting a default of ADP to ATP regeneration in that strain.

On the opposite, at later incubation times, when antibiotics were produced, a more active degradation of the polyphosphate pool was observed in the *ppk* mutant than in the wild type strain (A. Smirnov, unpublished results). Since, a super-induction of *phoR/phoP* expression was previously shown to take place in the *ppk* mutant strain (Ghorbel et al, 2006b), genes belonging to the *pho* regulon are likely to be over-expressed in this mutant. The over-expression of genes encoding polyphosphate phosphatases activities, likely to belong to the *pho* regulon, is thought to responsible for the fast degradation of the polyphosphate pool in that strain. Furthermore, we demonstrated that in these conditions, at very late incubation times, the intracellular concentration of ATP was higher in the *ppk* mutant strain than in the wild type strain (C. Esnault, unpublished results).

These experimental data led us to propose a novel model of the regulation of antibiotic biosynthesis that suggests that, in *Streptomyces*, the real trigger of antibiotic biosynthesis might be a deficit in the energetic charge of the cell.

A Novel View Of The Regulation Of Antibiotic Biosynthesis

The model



This novel model predicts that, in an attempt to re-establish its energetic balance, a strong activation of central metabolism will take place, in the *ppk* mutant strain. Central metabolic pathways coupled to the respiratory chain are indeed the main ATP producing route within the cell. This activation will lead to the production of the necessary ATP, of carbon skeletons and of reduced cofactors (NADH). In this situation the carbon skeletons generated by this activation will not be used for biomass construction but will constitute "building blocks" for antibiotics biosynthesis. It has, indeed, long been known that, in *Streptomyces*, antibiotic production occurs in the period of slow or no growth (weak anabolism).

Furthermore, this stimulation of central metabolism is predicted to yield abundant reduced co-factors that should be re-oxidized by the respiratory chain. However, if the amount of reduced co-factors produced exceeds the oxidative capability of the respiratory chain, electron leakage and thus the generation of reactive radicals (ROS, RNS) will take place leading to an increase of internal oxidative stress that was observed (see below).

Experimental validation of the model

The experimental validation of this model is in process by physiologic, transcriptomic, proteomic and metabolomic approaches.

However, we have already demonstrated that the *ppk* mutant takes up glucose (H. Hamdali et al., unpublished results) and consumes its internal polyphosphate pool (A. Smirnov et al., unpublished results) more actively than the wild type strain. Since glucose and phosphate are the needed fuels of metabolism, their enhanced consumption is indeed a sign of the activation of the metabolism.

Furthermore, the enhanced sensitivity to external oxidative stress (H2O2) of the *ppk* mutant in condition of antibiotic production (Ghorbel et al., 2006b) correlates with an enhanced internal oxidative stress that is likely to result from the stimulation of central metabolism. Since oxidative stress can be potentially damaging for the cell, the bacteria has to precisely regulate the delicate balance between the necessary stimulation of its central metabolism, to produce the indispensable ATP, and the potentially deleterious effects of the oxidative stress resulting from this stimulation. Some of our results suggest the existence of mechanisms sensing internal oxidative stress and reducing the activation of central metabolism (and thus the production of antibiotic) when the internal oxidative stress raises too dangerously (Nowacka-Mazurek et al., unpublished results).

At last, preliminary results of still on going proteomic studies of the *ppk* mutant strain indeed confirmed the over-expression of enzymes involved in the resistance to oxidative stress (catalases, superoxide dismutases). More importantly, these studies revealed the over-expression of some key enzymes of glycolysis and Krebs cycle. The over-expression of these enzymes is thought to be responsible for the over-production of some metabolites that constitute precursors for antibiotic biosynthesis. Furthermore, enzymes of the respiratory chain were also shown to be over-expressed indicating an active respiration related to the strong activation of central metabolism.

Conclusion

The novel model proposed for the regulation of antibiotic biosynthesis suggests that a weak energetic state might be the real trigger of antibiotic biosynthesis. A strong induction of the expression some specific enzymes of central metabolism is taking place in condition of energetic deficit. The over-expression of these specific enzymes is thought to lead to the accumulation of the carbon skeletons constituting precursors of antibiotics biosynthesis. This model, clarifies the nature of the relations existing between primary and secondary metabolism. It has quite a high explicative and predictive value and is easily testable.

Companies wish to be able to produce novel bio-active molecules with high titters. To do so, it is first necessary to identify and clone novel biosynthetic pathways. It is then necessary to increase their expression by genetically engineering genes encoding specific or pleiotropic regulators either in the producing strain or in a super-host. However, in any cases, it will be necessary to increase the precursors supply within the cell. To achieve this, it is of fundamental importance to get a better understanding of the regulation of central metabolism, the precursors supplier, in Actinomycetes species. The novel model proposed should inspire the design of rational strategies to increase the supply of antibiotics precursors and thus antibiotic production in Actinomycetes species. It can thus contribute to enhance the production of novel molecules previously too weakly produced to be detectable and thus tested for biological activity.

Companies are also sometimes facing the problematic genetic instability of industrial over-producing strains. The high producing strains derive quickly and their producing abilities are sometimes reduced or lost, generation after generation. The deleterious effects of an high oxidative stress that is an obligate consequence of an elevated antibiotic production might be responsible for this instability. It is thus also important to get a good knowledge of the different strategies used by the strains to cope with oxidative stress in order to construct stable hyper producing strains.

References

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