Dissecting the role of the two *Streptomyces peucetius* var. *caesius* glucokinases in the sensitivity to carbon catabolite repression

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In memory of Prof. Arnold L. Demain, an outstanding scientist, a good mentor and a best friend.

Abstract: Streptomyces peucetius var. *caesius*, the doxorubicin-producing strain, has two glucokinases (Glks) for glucose phosphorylation. One of them (ATP-Glk) uses adenosine triphosphate as its phosphate source, and the other one uses polyphosphate (PP). Glk regulates the carbon catabolite repression (CCR) process, as well as glucose utilization. However, in the streptomycetes, the specific role of each one of the Glks in these processes is unknown. With the use of PP- and ATP-Glk null mutants, we aimed to establish their respective role in glucose metabolism and their possible implication in the CCR. Our results supported that in *S. peucetius var. caesius*, both Glks allowed this strain to grow in different glucose concentrations. PP-Glk seems to be the main enzyme for glucose metabolism, and ATP-Glk is the only one involved in the CCR process affecting the levels of α-amylase and anthracycline production. Besides, analysis of Glk activities in the parental strain and the mutants revealed ATP-Glk as an enzyme negatively affected by high glucose concentrations. Although ATP-Glk utilizes only ATP as the substrate for glucose phosphorylation, probably PP-Glk can use either ATP or polyphosphate. Finally, a possible connection between both Glks may exist from the regulatory point of view.

Keywords: *Streptomyces peucetius* var. *caesius*, Glucokinases, Carbon catabolite repression, Anthracyclines, α-amylase

Introduction

Glucokinase (Glk) is a key enzyme for the glucose catabolism in bacteria since it catalyzes its phosphorylation to produce the intermediary glucose 6-phosphate (Romero-Rodríguez et al., 2015a). Phosphorylation by Glk occurs with the use of ADP, ATP, or inorganic polyphosphate (PP) as the phosphate donors (Kawai et al., 2005).

*Streptomyces peucetius* var. *caesius* overproduces doxorubicin, a drug utilized for breast cancer treatment. This bacterium comes from a round of mutagenesis of the wild-type strain *Streptomyces* peucetius with N-nitroso-N-methyl urethane. The wild-type strain mainly produces daunorubicin, another antitumor that is in disuse due to its cardiotoxicity. *S. peucetius* var. *caesius* contains two Glks, one utilizes ATP as the donor of the phosphate group (ATP-Glk) (Imriskova et al., 2005), and the other uses inorganic PP (PP-Glk) (Ruiz-Villafán et al., 2014). The PP-Glk activity is induced by glucose in this microorganism, and its activity levels are 1.8 times higher than those from ATP-Glk. The high PP-Glk activity levels are observed only in *S. peucetius var. caesius* but not in its parental *S. peucetius* strain, nor other streptomycetes like *Streptomyces coelicolor*, *Streptomyces thermocarboxydus*, and *Streptomyces lividans* (Ruiz-Villafán et al., 2014).

Initial reports on both Glks indicate that they occur in the chlortetracycline (CTC) producer, *Streptomyces aureofaciens* (Hořálek et al., 1976). In this microorganism, the ATP-Glk is expressed mainly during the exponential growth phase and the PP-Glk during the stationary phase, correlating with CTC production (Hořálek et al., 1976). Although described in several actinobacteria (Hsieh et al., 1996; Koide et al., 2013; Lindner et al., 2010; Tanaka et al., 2003), there is still little knowledge about the PP-Glk significance in the streptomycetes physiology.

In addition to glucose phosphorylation, in the genus *Streptomyces*, the Glk has been involved in other physiological processes. Therefore, the enzyme regulates the carbon catabolite repression (CCR) (Angell et al., 1994), as well as the glucose incorporation and utilization (Romero-Rodríguez et al., 2015b; van Wezel et al., 2005). Besides, a regulatory role in the metabolism of carbon sources different from glucose also seems to be due to Glk (Gubbens et al., 2012; Guzmán et al., 2005; Kwakman & Postma, 1994). In addition to its effects on the central metabolism, Glk also plays a significant role in regulating secondary metabolite production (Escalante et al., 1999; Gubbens et al., 2012; Romero-Rodríguez et al., 2016).

The multiple functions described for Glk raised the question about the differential role of ATP-Glk and PP-Glk in some of these events in *Streptomyces*. Therefore, by using ATP-Glk and PP-Glk null mutants, this work aimed to understand each enzyme’s participation in the glucose metabolism, CCR, and the synthesis of anthracyclines in *S. peucetius* var. *caesius*.

Materials and Methods

Bacterial Strains and Culture Conditions

The *S. peucetius* var. *caesius* NRRL B-5337 strain was kindly donated by the Agriculture Research Service Culture Collection, US Department of Agriculture (Peoria, IL). The ATPglk and PPglk null mutants were constructed from the parental strain *S. peucetius* var. *caesius*...
as recently described (Rocha et al., 2018). These mutants are available at the UNAM-48/WFCC (Mexico City). The strains were stored in vials with glycerol (20%, vol/vol) at −70°C. Mycelia from these strains were obtained as previously reported and conserved in YMG medium (Ruiz-Villafán et al., 2014).

Submerged cultures were performed in 250-ml baffled Erlenmeyer flasks containing 50 ml media and incubated at 30°C on a rotary shaker at 200 rpm. Initially, flasks containing YFM medium (50 ml) were inoculated with the strain cell suspension equivalent to 8 mg dry cell weight (dcw) mycelium and pregrown for 8 to 10 h. After this time, the culture was washed twice with a volume of isotonic salt solution (ISS), and an equivalent of 2 mg dcw used as the fermentation inoculum.

For growth, enzyme determinations, and anthracycline production, fermentations were performed in NDYE medium containing 10 mM NaNO₃, 1 mM K₂HPO₄ 3H₂O, 0.5 mM MgSO₄ 7H₂O, and 0.2% yeast extract. This medium was supplemented with trace elements, vitamin solution (Dekleva et al., 1985), and the desired carbon source (50 to 300 mM).

**Growth and Glucose Consumption**

At desired times, cells were collected by centrifugation (10 000 × g for 10 min at 4°C) from 10 ml fermentation samples. Growth and glucose remaining in the culture medium were determined as previously described (Ruiz-Villafán et al., 2014).

**Starch Analysis**

A colorimetric reaction with iodine measured the residual starch in the medium, and its absorbance was read at 620 nm. The reaction contained 0.1 ml of the appropriate dilution of the culture medium supernatant, mixed with 2.4 ml of an iodine solution containing 30 g/l I and 3 g/l I₂, diluted to 4% (vol/vol). For calculations, the results were compared to a standard starch curve containing between 0 and 12 g/l (Giraud et al., 1993).

**Protein Analysis and Glucose Kinase Activities**

Cells were recovered by centrifugation at 10 000 × g for 10 min and 4°C. After two consecutive washes with one ISS volume, they were resuspended in 1 ml 50 mM Tris–HCl, pH 7.2, containing Protease Inhibitor Cocktail Powder (Sigma-Aldrich) following the manufacturer suggestion. Cytosolic extracts were obtained by sonicating the cells with four pulses of 50 W, during 1 min with 30 s of an interval between each pulse. After centrifugation (13 000 × g for 10 min at 4°C), each cytosolic fraction was recovered for protein and enzyme activity assays (Ruiz-Villafán et al., 2014).

Glucokinases were assayed, using 50 µl of the cytosolic extract, measuring the NADPH formation at 340 nm through a Glk-glucose 6-phosphate dehydrogenase (Roche) coupled reaction as previously reported (Ruiz-Villafán et al., 2014). ATP was used as the substrate to measure ATP-Glk activity and inorganic PP for PP-Glk. Appropriate dilutions of the cytosolic extracts were used for protein evaluation, employing the Bradford reagent and bovine serum albumin as the standard (Bio-Rad).

**Amylase Activity**

Amylase activity assays used the strain culture supernatant samples obtained during the 96 h fermentation. Enzyme activity was measured using the Randox amylase assay kit (Randox Laboratories, Antrim, UK), measuring the released p-nitrophenol at OD₄₀₅nm with a molar extinction coefficient of 18.5 mM⁻¹ cm⁻¹ (Lorentz, 2000). For reaction, 50 µL of the culture medium supernatant was mixed with 500 µL of the reagent, incubated at 37°C, and the absorbance measured every minute for a 10-min period. A unit of amylase activity (U) was defined as the nmol of p-nitrophenol released in a min per mg of protein.

**Anthracycline Determination**

Cell cultures from different incubation times were centrifuged (10 ml at 10 000 × g for 10 min), and the recovered cell pellet washed twice with one volume of ISS. Anthracyclines were extracted by cell sonication in a mixture of 0.05 M acetone-sulfuric acid (4:1). The resulting anthracyclines were measured at 495 nm, using a molar extinction coefficient of 11 500 M⁻¹ cm⁻¹ (Ruiz-Villafán et al., 2014).

**Results**

**Role of the Two Glks in the Glucose Metabolism and Anthracycline Formation**

To determine the participation of the Glks in glucose metabolism and secondary metabolite formation, we determined the effect of three different sugar concentrations (50, 100, and 300 mM) on biomass formation, glucose consumption, glucose kinase activity, and anthracycline formation in all the strains.

**Growth and glucose consumption**

Fig. 1a shows the maximum biomass reached by the three strains at different glucose concentrations. Regarding the parental and the PP-Glk null mutant, the biomass production values were lower in the ATP-Glk null mutant at all the tested glucose concentrations. As shown in Fig. 1a, b, c, d, the parental strain consumed all the glucose present at 50- and 100-mM sugar concentrations. However, only 60% of the sugar was consumed by this strain at 300 mM glucose. The ΔPPΔglk mutant showed less ability to consume glucose, especially at 100- and 300-mM glucose. In this last concentration, this strain consumed only 32% of the sugar. Besides, the ATP-Glk null mutant was severely affected in its glucose consumption as only around 50 mM of the sugar was consumed in all tested sugar concentrations, and the slope of the plot consumption was markedly lower than the other strains, supporting that both Glks are required to metabolize glucose.

**Glucose kinase activities**

As reported by Ruiz-Villafán et al. (2014) for the S. peucetius var. caesius strain, PP-Glk activity levels were greater than those of ATP-Glk (Fig. 2a,b). Accordingly, the parental strain and the ΔATPΔglk exhibited the highest PP-Glk activity (around 400 U/mg protein) at 24 h incubation. As expected, when PP-Glk activity was measured in the ΔPPΔglk (Fig. 2a), it showed a basal and reduced activity (5%) at any of the tested glucose concentrations.

Regarding the ATP-Glk (Fig. 2b), a maximum activity value was observed in the parental strain at 50 mM glucose concentration (around 300 U/mg protein), which decreased at higher glucose concentrations. Although no activity was expected in the ΔATPΔglk, it showed high basal activity at all glucose concentrations tested. Unexpectedly, reduced ATP-Glk activity was also observed in the ΔPPΔglk with a maximum reduction at 300 mM glucose. These activity levels were even lower than those observed in the ΔATPΔglk. An explanation for this result is that PP-Glk may have some ATP-Glk activity, thus, in PP-Glk null mutants, the total ATP-Glk activity could be affected. Another possibility is that both enzymes interact with each other; therefore, in PP-Glk deleted mutants, the affinity of ATP-Glk for its substrate decreases.
Fig. 1. (a) Effect of different glucose concentrations over maximum biomass production by the *Streptomyces peucetius* var. *caesius* parental strain (black bars), ΔATPglk (white bars), and ΔPPglk (striped bars). Glucose consumption in NDYE media with 50 mM (b), 100 mM (c), and 300 mM (d) glucose by the *S. peucetius* var. *caesius* parental strain (filled circle), ΔATPglk (open square), and ΔPPglk (open triangle).

Fig. 2. PP-glucose kinase (a) and ATP-glucose kinase (b) specific activities of the *Streptomyces peucetius* var. *caesius* parental strain (black), ΔATPglk (white), and ΔPPglk (striped) mutants grown at different glucose concentrations. Both activities were determined at 24 h incubation in all sugar concentrations.

Anthracycline production
When anthracycline production was evaluated in these strains (Fig. 3a, b, c), an evident inhibitory effect was displayed by glucose on the parental strain and the PP-Glk null mutant. The parental strain was inhibited at 100 mM glucose, but not 50 mM, and the PP-Glk was marginally inhibited at 300 mM, but not at 50 nor 100 mM. Although the concentration of anthracyclines in the ATP-glk null mutant is lower than those of the other two strains, antibiotic levels remained practically the same in all glucose concentrations.
Influence of ATP- and PP-Glk Mutations on Starch Consumption, Amylase Activity, and Anthracycline Formation

Starch-hydrolyzing enzymes are widely distributed in several Streptomyces species, and their amylase production is sensitive to CCR (Flores et al., 1993; Virolle & Bibb, 1988). To evaluate the Glks participation in the sensitivity of α-amylase production to CCR in *S. peucetius* var. *caesius*, the parental and its derived Δglk strains were analyzed.

Initially, we evaluated these strains for their ability to consume starch and determined the glucose effect on this variable. All the strains grew well in minimal medium supplemented with 1% starch as the only carbon source. The ΔATPglk completely consumed starch at 30 h incubation, while the parental and ΔPPglk strain required 18 additional hours to utilize this carbon source (Fig. 4a). However, the presence of 300 mM glucose prevented starch consumption in the parental strain and the ΔPPglk (Fig. 4b) but not in the ΔATPglk. According to van Wezel et al. (2007), the ATP-Glk enzyme is involved in glucose transport by interacting with its transporter, the GicP protein. Thus, in the absence of ATP-Glk, there should be a decrease in glucose input. In this sense, the ΔATPglk mutant consumed only 15% of the 300 mM glucose in the culture medium. In addition, the consumption slope is also markedly lower than the slopes corresponding to the parental strains and ΔPPglk, which supports their difficulties in the consumption of glucose by this strain (Fig. 1d).

When amylase production was determined in the starch medium, we found a maximum value of enzyme activity in the above strains at 36 h incubation (Fig. 5a). Although dissimilar, these enzyme levels allowed an efficient starch utilization by the parental and mutant strains (Fig. 4a). Under these conditions, we detected higher amylase activity in the ATP-glk null mutant with enzyme levels almost sixfold higher than the parental and PP-glk null mutant (Fig. 5a). In this context, only a minor inhibition of enzyme production was observed in the ΔATPglk when added glucose to the medium. On the contrary, concerning the starch medium, a strong inhibition of enzyme activity was exerted by glucose (around 10-fold) in the parental strain and its ΔPPglk derivative (Fig. 5a). These low enzyme levels prevented the strains from efficient starch consumption (Fig. 4b). A specific regulator (reg1) for the amylase genes *amlB* and *amy* in *S. lividans* was reported by Nguyen (1999). Mutants lacking this regulator are insensitive to the negative glucose effect on amylase expression. It has been speculated that only the enzyme ATP-Glk exerts its regulation through specific regulators such as *reg1* (Mahr et al., 2000). If that were the case, then the PP-Glk enzyme cannot regulate those transcriptional factors like ATP-Glk does.

Anthracycline production by *S. peucetius* var. *caesius* is subject to CCR (Escalante et al., 1999). Therefore, we also determined the
Table 1. Effect of Glucose on Biomass Production and Anthracycline Formation in Starch Growing Cells

|                  | Biomass (mg/ml) | Anthracyclines (μg/mg mycelium) |
|------------------|----------------|---------------------------------|
|                  | Starch         | Starch + glucose                | Starch         | Starch + glucose                |
| Parental         | 2.1 ± 0.183    | 2.8 ± 0.164                    | 20 ± 1.35      | 1.15 ± 0.157                   |
| ΔATP glk         | 1.8 ± 0.316    | 1.9 ± 0.241                    | 25 ± 2.64      | 27 ± 0.632                     |
| ΔPP glk          | 2.3 ± 0.249    | 3.2 ± 0.134                    | 30 ± 1.74      | 0.98 ± 0.075                   |

*Cells were grown for 36 h in NDYE medium with 1% starch and starch plus 300 mM glucose.

Effect of Different Carbon Sources on Growth and Anthracycline Formation by the Parental Strain and Its Two Glk Null Mutants

We determined the relevance of Glk participation on the utilization of other carbon sources in the parental strain and its Glk null mutants. For this purpose, we evaluated the maximum biomass formation and anthracycline production.

As seen in Table 2, compared to the parental strain, in 100 mM of the tested carbon sources, the mutants reached a higher final biomass production. This behavior contrasted with that observed when the mutants were grown in the presence of 100 mM glucose (Fig. 1c). Regarding anthracycline production, its levels increased in all tested carbon sources in the ATP-Glk null mutant. On the contrary, except glycerol, the PP-Glk null mutant produced lower specific anthracycline production levels in all tested carbon sources (Table 2). Surprisingly, higher anthracycline levels were observed in this ΔPP glk strain grown in glycerol.

Discussion

S. peucetius var. caesius has two functional Glks, which allow the strain to grow in the presence of high glucose concentrations (Ruiz-Villafán et al., 2014).

Removal of one of the two glk genes (ATP- or PP glk) allowed the mutant strains to grow in glucose as the only carbon source but reduced the total glucose consumption and biomass formation; thus, supporting the necessity of the two glucokinases to metabolize the sugar.

The in vitro Glk activity values exhibited by the parental strain were higher for PP-Glk than ATP-Glk, suggesting PP-Glk as the main enzyme in S. peucetius var. caesius for glucose metabolism. ATP-Glk was proportionally reduced as a function of the glucose concentration present in the culture medium, and this reduction was not observed for PP-Glk. The specific negative effect of glucose on the ATP-Glk activity has not been previously reported for other microbial Glks and suggested a differential role for both enzymes.

Regarding the enzyme’s role in CCR, our results have shown a sensitivity of anthracycline formation to CCR in the parental strain and the ΔPP glk mutant but not in the ΔATP glk mutant. These phenotypes were proportional to the sugar concentration present in the culture medium, thus, reinforcing ATP-Glk as the only enzyme involved in this regulatory effect.

A more evident effect of glucose on anthracycline formation (Fig. 5b) was apparent by growing these strains in a starch medium. It is noteworthy to mention that regarding the parental strain, a 15% and 30% decrease in the growth of the ATP-Glk null mutant was observed when these strains were grown in starch or starch plus glucose media, respectively.

The different glucose concentrations used in the parental strain and its Glk null mutants as the sole carbon source can explain the differences observed in the results. In the parental strain, glucose was used as the sole carbon source, and the ΔATP glk strain was grown in starch, while the ΔPP glk strain was grown in glycerol.

In summary, the results presented here suggest that Glk is a key enzyme in S. peucetius var. caesius for glucose metabolism and anthracycline formation, and its participation is essential for the strain to grow in the presence of high glucose concentrations.
Table 2. Effect of Different Carbon Sources on Maximum Growth and Anthracycline Production by the Streptomyces peucetius var. caesius Parental and its ΔATPglk and ΔPPglk Mutants

| Carbon source (100 mM) | Strain     | Biomass (mg/ml) | Anthracyclines (μg/mg mycelium) |
|------------------------|------------|-----------------|---------------------------------|
| Xylose                 | Parental   | 1.85 ± 0.21     | 6.7 ± 0.65                      |
|                        | ΔATPglk    | 4.55 ± 0.17     | 9.3 ± 0.50                      |
|                        | ΔPPglk     | 3.06 ± 0.28     | 3.8 ± 0.22                      |
| Galactose              | Parental   | 2.10 ± 0.20     | 13.1 ± 0.57                     |
|                        | ΔATPglk    | 3.75 ± 0.13     | 17.7 ± 0.22                     |
|                        | ΔPPglk     | 2.70 ± 0.35     | 7.9 ± 0.61                      |
| Fructose               | Parental   | 2.23 ± 0.25     | 9.3 ± 0.35                      |
|                        | ΔATPglk    | 3.83 ± 0.34     | 10.0 ± 1.06                     |
|                        | ΔPPglk     | 3.96 ± 0.35     | 7.8 ± 0.32                      |
| Glycerol               | Parental   | 2.38 ± 0.19     | 8.4 ± 0.76                      |
|                        | ΔATPglk    | 3.55 ± 0.27     | 10.7 ± 0.24                     |
|                        | ΔPPglk     | 4.73 ± 0.42     | 15.6 ± 1.80                     |

Although no ATP-Glk activity was expected in the deleted ATPglk mutant, the basal activity detected may reflect either the presence of additional hexokinases or the ability of PP-Glk to use ATP as substrate (Fig. 2b). PP-Glk activities with both specificities (for PP and ATP) have also been reported in actinobacteria like S. coelicolor (Koide et al., 2013), Corynebacterium glutamicum (Lindner et al., 2010), and Mycobacterium tuberculosis (Hsieh et al., 1996).

On the contrary, when we determined the ATP-Glk activity levels in the ΔPPglk mutant, a 40% to 70% reduction in the enzyme levels was observed (Fig. 2b). Since van Wezel et al. (2007) proposed that ATP-Glk interacts with the glucose transporter GlcP, the reduction in enzyme activity could be due to lack of ATP-Gulch, which is needed for the transport. Another explanation is that both Glks interact each other and regulate their activity in this way.

The previously mentioned binding between ATP-Glk and the GlcP transporter to internalize and phosphorylate glucose may explain the limited consumption of glucose observed in the ΔATPglk. However, at 500 mM glucose, the ΔPPglk mutant also showed a decrease in the internalized glucose. Regardless of the transport, the phosphorylation rate will depend on the Glk affinity for glucose of each enzyme. The biochemical characterization of ATP-Glk and PP-Glk of S. coelicolor reported Km values for glucose of 1.4 mM (Imriskoiva et al., 2001) and 12 μM (Koide et al., 2013), respectively. A similar Km value for glucose (1.6 mM) was reported for the ATP-Glk from S. peucetius var. caesius (Imriskoiva et al., 2005). The PP-Glk of S. peucetius var. caesius has not yet been characterized. Still, considering the reported enzyme characteristics of S. coelicolor, it would be possible to explain why the ΔATPglk mutant consumes less glucose, as this enzyme is saturated with the available high glucose concentration.

We found that both Glks seem to play a global role in using other carbon sources, even when they are not metabolized through Glk. Thus, the mutants generated higher biomass than the parental strain in all tested carbon sources. The anthracycline production levels were higher in the ATP-Glk null mutant. Although both mutants showed an increase in anthracycline production in glycerol, the PP-Glk null-mutant was the best producer. For years, the doxorubicin biosynthetic pathway has been analyzed to understand its synthesis and regulation. This knowledge elicited culture condition modifications or allowed mutational programs to increase this compound production levels for clinical use (Niraula et al., 2010). Thus, our finding that the glycerol-grown ΔPP-glk mutant produces high anthracycline titers is of great relevance for its production on an industrial scale. Regarding these carbohydrates, there are earlier reports on a similar unpressed phenotype for enzymes involved in the use of glycerol, lactose, and xylose in ATP-Glk null mutants of S. coelicolor (Kwakman & Postma, 1994) and S. peucetius var. caesius (Segura et al., 1996), grown in glycerol.

In conclusion, in S. peucetius var. caesius, both Glk activities maintain a basal flow of metabolites through glycolysis for growth and secondary metabolites production. From them, only ATP-Glk appears to participate in the CCR process. Looking at the ATP-Glk amino acid sequence, a lack of DNA-binding motifs in this enzyme is detected. Therefore, as recently suggested (Ruiz-Villafán et al., 2021), its action mechanism likely requires additional proteins or cofactors to be performed. In S. coelicolor, even though the ATP-Glk also lacks DNA-binding motifs, the enzyme specifically affects the expression of 43 genes by a mechanism that awaits to be defined (Romero-Rodríguez et al., 2016). Recently, the report of Glk crotonylation by an acyltransferase opened a plausible mechanism to explain how the modified ATP-Glk may interact with its corresponding transcriptional factors (Martín et al., 2021; Sun et al., 2020).

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Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest

We declare that we do not have any commercial or associative interest representing a conflict of interest connected with the work submitted.

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