

Research Paper

Yeast HXK2 gene reverts glucose regulation mutation of penicillin biosynthesis in *P. chrysogenum*

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Abstract

The mutant *Penicillium chrysogenum* strain *dogR5*, derived from strain AS-P-78, does not respond to glucose regulation of penicillin biosynthesis and β -galactosidase, and is partially deficient in D-glucose phosphorylating activity. We have transformed strain *dogR5* with the (hexokinase) *hvk2* gene from *Saccharomyces cerevisiae*. Transformants recovered glucose control of penicillin biosynthesis in different degrees, and acquired a hexokinase (fructose phosphorylating) activity absent in strains AS-P-78 and *dogR5*. Interestingly, they also recovered glucose regulation of β -galactosidase. On the other hand, glucokinase activity was affected in different ways in the transformants; one of which showed a lower activity than the parental *dogR5*, but normal glucose regulation of penicillin biosynthesis. Our results show that *Penicillium chrysogenum* AS-P-78 and *dogR5* strains lack hexokinase, and suggest that an enzyme with glucokinase activity is involved in glucose regulation of penicillin biosynthesis and β -galactosidase, thus signaling glucose in both primary and secondary metabolism; however, catalytic and signaling activities seem to be independent.

Key words: glucose regulation, glucokinase, penicillin, *Penicillium chrysogenum*.

Introduction

Glucose is an important regulator of metabolism and gene expression, and organisms have developed a sophisticated genetic and biochemical program to respond to the presence of this readily usable carbon and energy source (Castillo *et al.*, 2006, Wang *et al.*, 2004). Carbon catabolite repression (CCR), which represses expression of genes involved in gluconeogenesis and catabolism of alternate carbon sources, is one of the better characterized regulatory circuits triggered by glucose (Gancedo, 1998).

The presence of glucose is sensed by eukaryotic cells in different ways (Rolland *et al.*, 2001, Santangelo, 2006), by membrane-bound receptors (with or without a transport function), and inside the cell by enzymes involved in its catabolism. Hexokinase enzymes play an essential role in glu-

cose sensing; in mammals, hexokinase IV regulates insulin release in pancreatic beta-cells (Efrat and Lodish, 1994), and in plants, hexokinases are involved in regulating expression of photosynthetic genes, seed germination, and ethylene signaling by glucose (Pego *et al.*, 1999, Sheen *et al.*, 1999, Yanagisawa *et al.*, 2003).

In *Saccharomyces cerevisiae*, the enzyme hexokinase 2 (Hvk2) has long been implicated in CCR (Entian and Mecke, 1982), a function initially attributed to its catalytic activity, but which is now thought to be carried out at the nuclear level, by interaction with other regulatory proteins like the broad domain transcriptional repressor Mig1 and the Snf1 kinase (Ahuatzi *et al.*, 2007). Hvk2 interacts with Mig1 when the yeast grows in high glucose concentration, preventing its phosphorylation at serine 311 by Snf1, thus

avoiding Mig1 nuclear export and derepression of genes subjected to CCR like *SUC2* (Ahuatzi *et al.*, 2007). Along with Hxk2, the most active glucose-phosphorylating enzyme in the yeast cell, two other enzymes are able to phosphorylate glucose in *S. cerevisiae*, hexokinase 1 (Hxk1) and glucokinase (Glk1) (Johnston and Carlson, 1992), which in turn have a role in other regulatory pathways triggered by glucose, like activation of glycolysis enzymes (Rolland *et al.*, 2001).

In contrast to the situation in *S. cerevisiae*, mutants isolated from the filamentous fungus *Aspergillus nidulans*, which lack hexokinase activity (*frA1*, “fructokinase” mutants) retained glucose CCR of ethanol and L-arabinose catabolism (Ruijter *et al.*, 1996). In a later study (Flipphi *et al.*, 2003), it was shown that double mutants of *A. nidulans*, impaired in both hexokinase (*frA1*, renamed *hxkA1*) and glucokinase (*glkA4*), are severely altered in CCR of three different test systems (ethanol, xylane and acetate utilization), whereas single mutants still retain a normal CCR function in response to D-glucose and D-fructose; therefore, the two hexose phosphorylating enzymes play parallel roles in glucose repression in *A. nidulans*.

Secondary metabolism in filamentous fungi is also subjected to glucose regulation. β -lactam biosynthetic genes have been shown to be repressed by glucose in the penicillin producers *Penicillium chrysogenum* (Feng *et al.*, 1994, Gutiérrez *et al.*, 1999) and *Aspergillus nidulans* (Espeso and Peñalva, 1992), and in the cephalosporin producer *Acremonium chrysogenum* (Jekosch and Kück, 2000). Glucose regulation of the penicillin genes in *A. nidulans* does not seem to be mediated by CreA, the transcriptional repressor mediating CCR (Espeso *et al.*, 1993); in contrast, strong evidence support a role for CreA in glucose regulation of the penicillin genes *pcbAB* and *pcbC* in *P. chrysogenum* (Fierro *et al.*, unpublished results) and for Cre1 in glucose regulation of the cephalosporin genes *pcbC* and *cefEF* (Jekosch and Kück, 2000). The knowledge about other components of the glucose-signaling pathway involved in glucose repression of β -lactam biosynthesis in fungi is very limited.

In *Penicillium chrysogenum* glucose, and to a lesser extent fructose, galactose and sucrose, cause a drastic reduction of penicillin titers (Revilla *et al.*, 1984). Glucose represses formation of δ -(L- α -aminoadipyl)-L-cysteiny-D-valine and isopenicillin N synthases (enzymes for the first and second steps of penicillin biosynthesis respectively), but not of penicillin acyltransferase (Martín *et al.*, 1999, Revilla *et al.*, 1986). Barredo *et al.* (1988) used N-Methyl-N'-nitro-N-nitrosoguanidine (47 μ M) to mutagenize germinating conidia of *P. chrysogenum* AS-P-78, and plated these conidia on minimal medium supplemented with lactose (27 mM) and D-2-deoxyglucose D-2-DOG (3 mM). D-2-DOG is a D-glucose analogue able to trigger CCR, it can be phosphorylated at C6 but not further metabolized, and thus it prevents growth of *P.*

chrysogenum AS-P-78 on minimal medium with lactose as sole carbon source. A mutant strain, *dogR5*, was isolated which could grow in this medium. As expected, strain *dogR5* was derepressed in β -galactosidase, and was deficient in phosphorylation of D-glucose and D-2-DOG, which suggested that it was deficient in glucokinase. In addition, strain *dogR5* did not respond to glucose regulation of penicillin biosynthesis and of isopenicillin-N-synthase activity (second step of penicillin biosynthesis), suggesting a common CCR pathway for both primary and secondary metabolism, a hypothesis supported by the results we have obtained in the present work.

In this study we report that *S. cerevisiae* *HXK2* gene is able to restore glucose regulation of penicillin biosynthesis and β -galactosidase in the *dogR5* mutant, and that hexokinase activity is absent from strains AS-P-78 and *dogR5* but is acquired after transformation with the *HXK2* gene. We present evidence that a glucokinase is an important component of the CCR pathway controlling formation of catabolic enzymes for alternate carbon sources and penicillin biosynthetic enzymes, thus participating in glucose signaling of both primary and secondary metabolism of *Penicillium chrysogenum*.

Materials and Methods

Strains and plasmids

Bacterial strains. *Bacillus subtilis* ATCC 6633 was used in bioassays for penicillin G quantification. *Escherichia coli* DH5 α was used as recipient for plasmid DNA transformation and amplification.

Fungal strains. *Penicillium chrysogenum* AS-P-78 was obtained in Antibióticos S.A. (León, Spain) by mutation from strains previously improved for penicillin production. It contains a tandem-arranged amplified genomic region, which includes the penicillin biosynthesis gene cluster (Fierro *et al.*, 1995).

Penicillium chrysogenum dogR5 was obtained after nitrosoguanidine mutation from strain AS-P-78 (Barredo *et al.*, 1988), it was selected for its growth on lactose-containing medium in the presence of D-2-deoxyglucose. It was kindly provided by Prof. J.F. Martín from the University of León (Spain).

Plasmid pULJB1 was constructed by introducing a 3.37 kb in *XhoI*-*ClaI* fragment from plasmid pSP73-HXK2, kindly provided by F Moreno (19), which contains the *S. cerevisiae* *HXK2* gene with its own promoter, into plasmid pULC43. pULC43, donated by S. Gutiérrez (University of León, Spain), is an integrative vector carrying a chloramphenicol-resistance gene for selection in *E. coli* and a phleomycin resistance cassette (*PpcbC::ble::Tcyc*) with the *ble* gene from *Streptoalloteichus hindustanus* as selection marker for fungal transformation.

Media and culture conditions

Media for bacteria

Standard LB and TB supplemented with the appropriate antibiotic were used for growth and selection of *E. coli* transformants. SOB, RF1 and RF2 media (Hanahan, 1983) were used to obtain competent *E. coli* cells. *Bacillus subtilis* spores were obtained by growing the bacteria in ME medium (g L⁻¹: peptone, 8; meat extract, 3; MnCl₂ 10⁻⁵ M, pH 7.2).

Fungal media

Complex medium for fermentation of *Penicillium* (MCFP) (g L⁻¹: lactose, 55; corn steep solids, 35; CaCO₃, 10; KH₂PO₄, 7; MgSO₄·7H₂O, 3; potassium phenylacetate, 4; pH 6.8). Complex agar medium for penicillin production (MCFP-A) (g L⁻¹: lactose, 50; Pharmamedia (Traders Protein, Forth Worth, TX, USA), 20; CaCO₃, 5; (NH₄)₂SO₄, 4; potassium phenylacetate, 4; agar, 20; pH 6.8). For both MCFP and MCFP-A, lactose was substituted by glucose at the same concentration in regulation experiments. *Penicillium* seed medium (MCIP) (g L⁻¹: sucrose, 20; corn steep solids, 20; yeast extract, 10; CaCO₃, 5; pH 5.7). Power sporulation medium was made by mixing (1:1, v/v) standard Czapek medium with PM1 (Fierro *et al.*, 1996). Protoplast MPPY medium was prepared as described (García-Rico *et al.*, 2007).

Sporulation of fungi. Spores from *P. chrysogenum* strains were obtained by growing the fungus on Petri dishes with Power medium and incubating at 25 °C for 5 to 7 days.

Growth with different carbon sources. *P. chrysogenum* strains were grown on the surface of 125 mL of Power solid medium in flasks for 7 days for spores production. Conidia were collected with 5 to 10 mL of saline solution (0.9% NaCl) together with 2 g of small sterile glass beads. Spores were released by gently rotating the flasks for 2 min, and the spore suspension decanted to a separate flask. Its concentration was calculated by means of a Neubauer chamber. For cultures in Petri dish, to test growth on different carbon sources, the spore suspension was diluted to get 10,000 conidia in 5 µL. This volume was inoculated with a micropipette on a point on the surface of Petri dishes containing Czapek minimal medium with different carbon sources (glucose, sucrose and lactose) at a final concentration of 3% w/v. D-2-deoxyglucose was added at a concentration of 3 mM to Czapek-lactose for regulation experiments.

Submerged culture fermentations of P. chrysogenum. Fifty mL of MCIP seed medium in 250 mL flasks were inoculated with 5 × 10⁶ spores mL⁻¹ and incubated at 25 °C for 36 h in an orbital shaker at 200 r.p.m. Five mL of this seed culture were then inoculated onto 250 mL flasks containing 50 mL of glucose- or lactose-containing MCFP medium, which were incubated at 25 °C for 120 h with constant 200 rpm. shaking. One mL samples were taken in

Eppendorf tubes from time 0 through 120 every 24 h, they were centrifuged (14000 r.p.m. for 10 min) and the supernatant was stored at -20 °C for quantification of penicillin G by bioassay. Mycelium samples were also taken every 24 h for enzymatic assays.

Bioassays and enzyme assays

Bioassays with top-agar

Petri dishes with 20 mL of glucose- or lactose-containing MCFP-A medium (2% agar) were inoculated at three points with 1 µL of a *P. chrysogenum* spore suspension (10³ spores) and incubated at 25 °C for 48 to 72 h. Once colonies were grown and beginning to sporulate, a top agar layer of molten TSA 1% agar, previously inoculated with 60 µL of a *Bacillus subtilis* spore suspension, was casted onto the MCFP-A medium, allowed to solidify for 15 min, and incubated at 30 °C for 16 h. Diffusion of penicillin into the medium causes inhibition halos in the growth of *Bacillus subtilis*, which are then measured to estimate penicillin G production.

Bioassays of samples from submerged culture fermentations

Quantification of penicillin G production of samples taken from submerged culture fermentations was performed as described (López-Nieto *et al.*, 1985).

Samples to determine enzyme activity (β-galactosidase, glucokinase and hexokinase) were obtained from submerged fermentations (shaking flask) in repressing conditions (glucose as carbon source) or non-repressing conditions (lactose as carbon source). Cultures were performed as described by López-Nieto *et al.* (1985).

Preparation of cell extracts and enzyme activity determination

Enzyme activities were determined in crude extracts of *P. chrysogenum*. Mycelium was filtered, washed (ice cold saline solution) and samples were frozen with liquid nitrogen, then grinded to obtain a fine powder that was re-suspended in cold extraction buffer containing: 50 mM potassium phosphate, 5 mM MgCl₂, 0.5 mM EDTA, 5 mM mercaptoethanol and pH 2. It was then centrifuged at 4000 rpm for 10 min; the supernatant was collected and centrifuged again at 10000 rpm for 5 min.

β-galactosidase activity was determined in crude extracts of *P. chrysogenum*, using the spectrophotometric method based on the hydrolysis of *O*-nitrophenyl-β-D-galactopyranoside (ONPG) (A₄₂₀) (Wallenfels 1962). Control reactions without ONPG were carried out in parallel.

Hexokinase activity was determined in 50 mM Pipes pH 7.5 in the presence of 5 mM MgCl₂, 5 mM D-fructose, 2 mM ATP, 0.5 mM NADP⁺, 2 U/mL glucose 6-P dehydrogenase and 4 U/mL of phosphoglucose isomerase at 30 °C, using a spectrophotometer (A₃₄₀; ε = 6.22 mM⁻¹ cm⁻¹). Absorbance increase per minute was de-

terminated in test and blank. The enzyme activity was quantified by taking into account that for each micromol of NADPH formed, there was an increase of 6.22 in the A_{340} (Panneman *et al.*, 1998).

Glucokinase activity was determined by a similar assay, except that 5 mM D-glucose was used as a substrate instead of fructose. Protein concentration in extracts was determined by the Bradford assay (BioRad).

ANOVA, and Minimal Significant Difference (95% confidence level) analysis were performed with SPSS19 software.

Penicillium chrysogenum dogR5 transformation

The protoplast method described by Cantoral *et al.* (1987), was followed for *P. chrysogenum* transformation. Selection of transformants was made with Czapek-sorbitol (1 M) minimal medium containing 30 $\mu\text{g mL}^{-1}$ phleomycin. Transformants grown in this medium were replicated on Czapek containing 40 $\mu\text{g mL}^{-1}$ phleomycin, and those growing at this antibiotic concentration were selected for analysis of penicillin production and enzyme assays.

Southern blot and hybridization

Integration of the pULJB1 plasmid, containing the *HXK2* gene, in the transformants was checked by hybridization. Total DNA of *P. chrysogenum* was obtained essentially as described by Specht *et al.* (Specht *et al.*, 1982). Southern blot of AlwN1 and/or *EcoRI*-digested DNA and hybridization were performed by standard procedures (Specht *et al.*, 1982). The 1.4 kb *HXK2* probe was obtained by digestion of pULJB1 with the restriction enzymes *NcoI* and *PstI*. The probe was labeled with [α - ^{32}P]dCTP by random primer (Ready-To-Go DNA Labeling Beads (Amersham). Detection of hybridizing signals was carried out by autoradiography, and copy number calculated by densitometric analysis, normalized by DNA concentration.

Results

The *Saccharomyces cerevisiae* *HXK2* gene reverts glucose regulation of penicillin biosynthesis in *Penicillium chrysogenum dogR5*.

Strains *P. chrysogenum* AS-P-78 and its derivative *dogR5* were first analyzed in a top-agar bioassay to test

their penicillin production in agar MCFP-A media with glucose (MCFP-A+G) or lactose (MCFP-A+L) as carbon source (Table 1).

Strain AS-P-78 produced clearly less penicillin (inhibition halo 38% smaller) with glucose than with lactose, whereas strain *dogR5* showed no significant differences in both media, indicating that it is deregulated with respect to the glucose regulation of penicillin biosynthesis.

Strain *P. chrysogenum dogR5* was transformed with plasmid pULJB1, containing the hexokinase encoding *HXK2* gene from *S. cerevisiae*. A total of 38 transformants were obtained, seven of which showed resistance to a phleomycin concentration of 40 $\mu\text{g mL}^{-1}$ and were selected for further experiments; they were named: T-I-6, T-II-1, T-II-3, T-III-3, T-IV-A, T-IV-1 and T-IV-5.

Using a more sensitive form of the top-agar bioassay screening (48 h incubation), it was found that apparently five of the transformants (T-I-6, T-II-1, T-II-3, T-III-3 and T-IV-A) had totally or partially recovered glucose regulation of penicillin biosynthesis, whereas transformant T-IV-1 did not show detectable production in either media and transformant T-IV-5 was not affected by transformation and retained the deregulated phenotype.

The transformants were then analyzed in a submerged culture fermentation to quantitatively test the degree of glucose regulation of penicillin biosynthesis recovered after transformation with the *HXK2* gene. Fermentations were carried out in flask cultures with MCFP medium containing glucose (MCFP+G) or lactose (MCFP+L) as carbon source (Figure 1).

Statistical analysis showed no differences between penicillin biosynthesis, by *dogR5* mutant, in both media. Conversely, ANOVA and MSD analysis showed significant difference at times at 24, 48 and 72 h ($p < 0.05$), of parental and transformants (except T-II-3 and T-III-3 mutants at 24 h, and T-II-1 at 48 h).

Strain AS-P-78 showed a strong glucose regulation throughout the fermentation, accumulating 7-fold more penicillin in MCFP+L than in MCFP+G after 120 h, whereas strain *dogR5* produced similar amounts in both media until 72 h, and accumulated only 25% more penicillin in MCFP+L at 120 h. In the *HXK2* transformants, different degrees of glucose regulation of penicillin biosynthesis

Table 1 - Glucose regulation of penicillin biosynthesis as measured by the inhibition halos produced by strains AS-P-78 and *dogR5* in a top-agar bioassay after 72 h of growth, and by submerged fermentation.

Strain	Medium	Inhibition halo (mm)*	Penicillin production in submerged cultures ($\mu\text{g/mL}$)
AS-P-78	MCFP-A+L	42.33 \pm 1.15	424.96 \pm 4.56
	MCFP-A+G	26.66 \pm 1.15	119.85 \pm 4.46
<i>dogR5</i>	MCFP-A+L	38.66 \pm 0.58	311.18 \pm 2.87
	MCFP-A+G	39.00 \pm 0.00	309.75 \pm 3.91

*Data are the average of three different experiments \pm standard deviation.

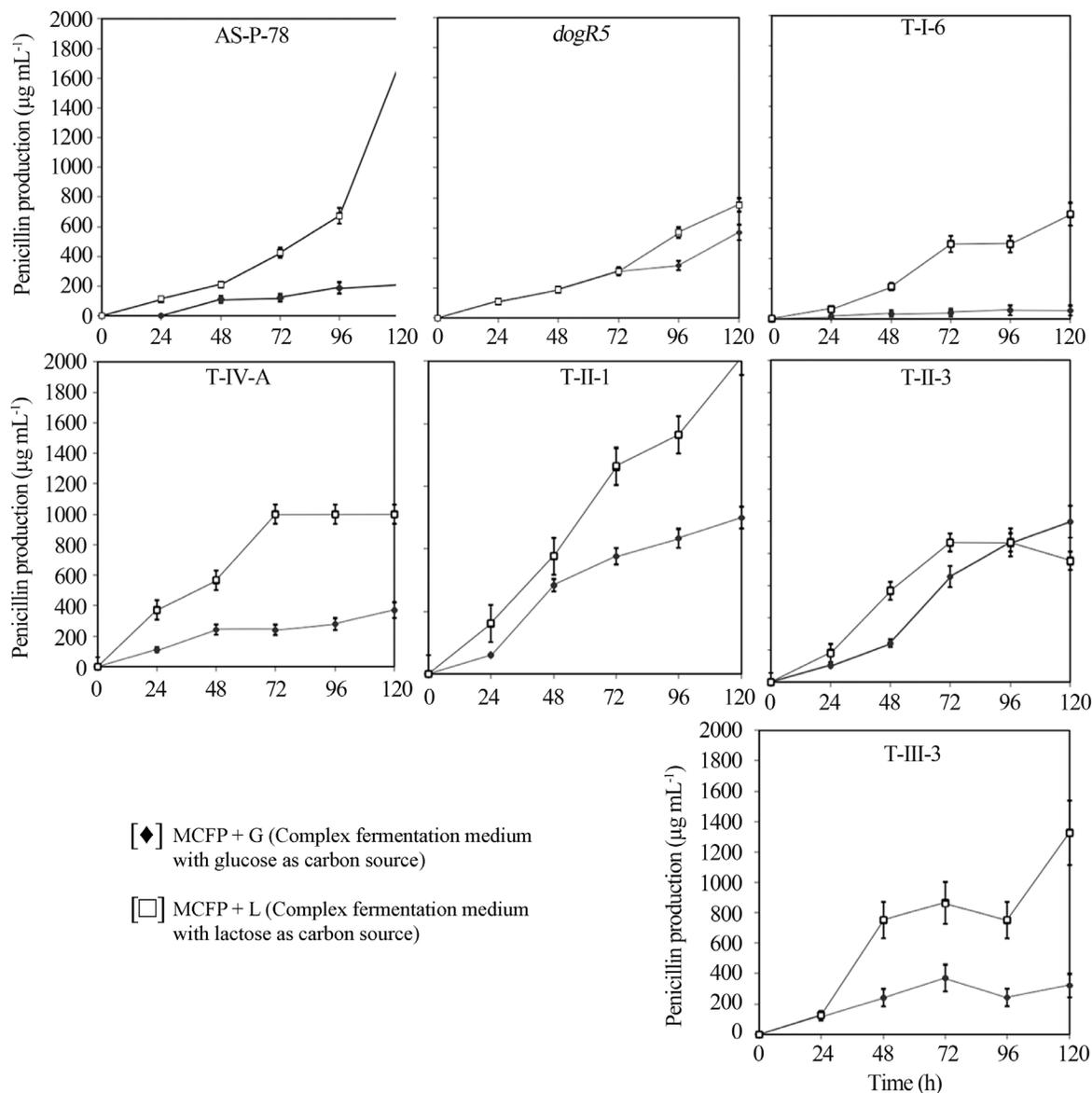


Figure 1 - Penicillin production in submerged cultures of strains AS-P-78, *dogR5*, and five of the *dogR5* transformants with the *HXK2* gene, in MCFP+G (complex fermentation medium with glucose as carbon source) and MCFP+L (complex fermentation medium with lactose as carbon source). Data correspond to the average of three repetitions from two independent experiments. Vertical bars represent standard deviations.

were observed; in the T-I-6 transformant regulation was fully restored, transformants T-III-3 and T-IV-A showed also a strong glucose regulation, which was less marked in transformant T-II-1. Transformant T-II-3 showed an important glucose regulation during the first 48 h of fermentation, but the accumulated penicillin in MCFP+G overcame that in MCFP+L at the end of the process. Therefore, four out of seven *HXK2* transformants clearly recovered glucose regulation of penicillin biosynthesis, and another one (T-II-3) showed a glucose regulation pattern during the first 96 h of cultivation.

To confirm the presence of the *HXK2* gene in the genome of the seven transformants, a Southern blot and hybridization with a *HXK2* probe was performed with *EcoRI*

and *AlwN1*-digested total DNA from parental strain and each of the transformants, and *EcoRI*-digested *S. cerevisiae* DNA. As shown in Figure 2, hybridization signals of the same size as in *S. cerevisiae* were obtained in all transformants, but were lacking in strains AS-P-78 and *dogR5*, even when the stringency conditions were lowered to facilitate heterologous hybridization. This result confirms the presence of the *S. cerevisiae HXK2* gene in the transformants, and shows that no gene with a high sequence similarity to *HXK2* is present in *P. chrysogenum* AS-P-78 and its derivative *dogR5*. Densitometric analysis indicated one gene copy was present in transformants T-I-6 and T-VI-A.

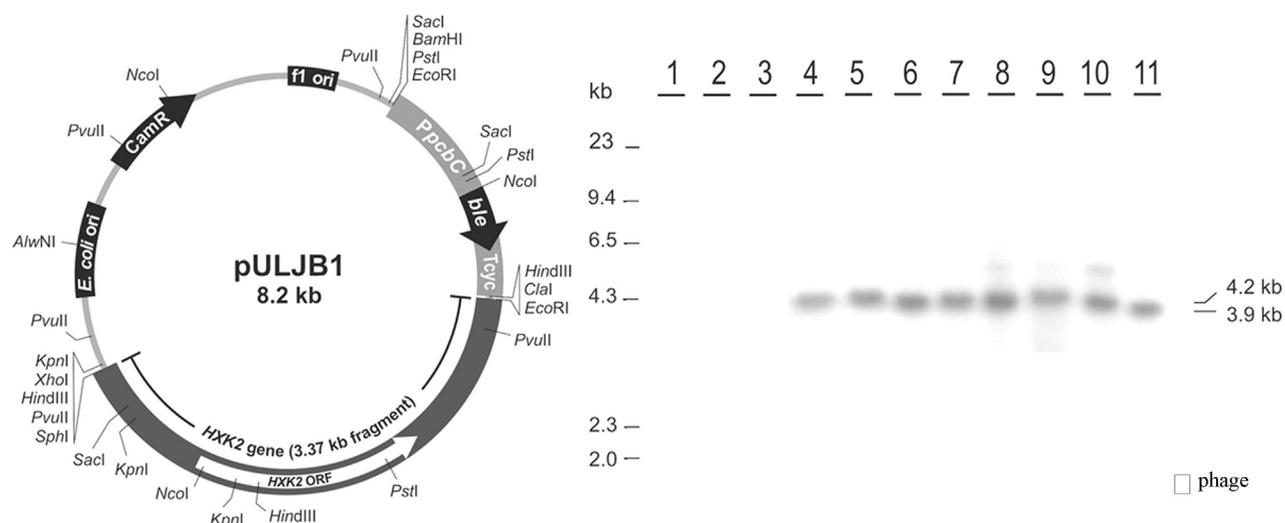


Figure 2 - Southern blot and hybridization with an *HXK2* probe of *EcoRI* and *AlwNI*-digested DNA from strains AS-P-78 (lane 2), *dogR5* (lane 3), T-I-6 (lane 4), T-II-1 (lane 5), T-II-3 (lane 6), T-III-3 (lane 7), T-IV-1 (lane 8), T-IV-A (lane 9), and T-IV-5 (lane 10). *EcoRI*-digested *Saccharomyces cerevisiae* DNA was used as positive control (lane 11). Indicated at the left is the size of DNA fragments belonging to a size marker (*Lambda DNA* digested with *HindIII*) loaded in lane 1.

Glucokinase activity is decreased in strain *dogR5* as compared to strain AS-P-78

The two transformants in which reversion of glucose regulation of penicillin biosynthesis was stronger, T-I-6 and T-IV-A, were chosen to analyze glucokinase and hexokinase activities, and compare them with activities in the parental *dogR5* and AS-P-78 strains. Samples from each strain, taken at 24 h intervals from fermentations in MCFP+G and MCFP+L media, were analyzed for both activities.

Results of glucokinase activity (Figure 3) showed that this activity is strongly regulated by the carbon source, being clearly higher in glucose-containing medium, especially in strain AS-P-78. Strain *dogR5* is partially deficient in glucokinase activity, having lost about half the activity in glucose medium. Glucokinase regulation by the carbon source is also altered in this strain, as differences of glucokinase activity in both carbon sources are much smaller than in strain AS-P-78.

Transformants T-I-6 and T-IV-A showed different behaviors as regard glucokinase activity; T-I-6 underwent an increase with respect to the parental strain *dogR5* in glucose medium, whereas in lactose medium the activity did not change substantially. However, transformant T-IV-A underwent a decrease of glucokinase activity as compared to strain *dogR5*, especially in glucose medium.

We can conclude that strain *dogR5* is partially deficient in glucokinase and altered in the carbon source regulation of this activity. Introduction of the *S. cerevisiae HXK2* gene in this strain caused modifications of the glucokinase activity, but of different nature depending on the transformant.

Hexokinase activity is absent in strains AS-P-78 and *dogR5* but was acquired by the *HXK2* transformants

Hexokinase activity was also analyzed in strains AS-P-78, *dogR5*, and the two transformants. As shown in Figure 4, hexokinase activity is absent in both *P. chrysogenum* AS-P-78 and *dogR5*, whereas transformants T-I-6 and T-IV-A have acquired a hexokinase activity after incorporation of the *S. cerevisiae HXK2* gene. This activity was similarly regulated in both transformants, being higher in glucose- than in lactose-containing medium throughout the fermentation.

The results obtained in the analysis of glucokinase, hexokinase, and penicillin production suggest that an enzyme with glucokinase activity is involved in glucose regulation of penicillin biosynthesis in *P. chrysogenum*, and that the *S. cerevisiae* hexokinase gene *HXK2* can complement the deregulated phenotype in the glucokinase-deficient strain *dogR5*.

Glucose regulation of β -galactosidase activity is recovered in the *HXK2* transformants

β -galactosidase is subjected to CCR, and strain *dogR5* is deregulated in the glucose control of this activity (3), so it was important to know if *HXK2* transformants were also able to recover this regulation. Analysis of β -galactosidase activity in submerged cultures with glucose- or lactose-containing MCFP medium (Figure 5) revealed that one transformant, T-I-6, fully recovered CCR of β -galactosidase to a level very similar to that in strain AS-P-78, with no activity in glucose medium but for the sample at 120 h. The other transformant, T-IV-A, partially recovered the regulation, maintaining from 20 through 120

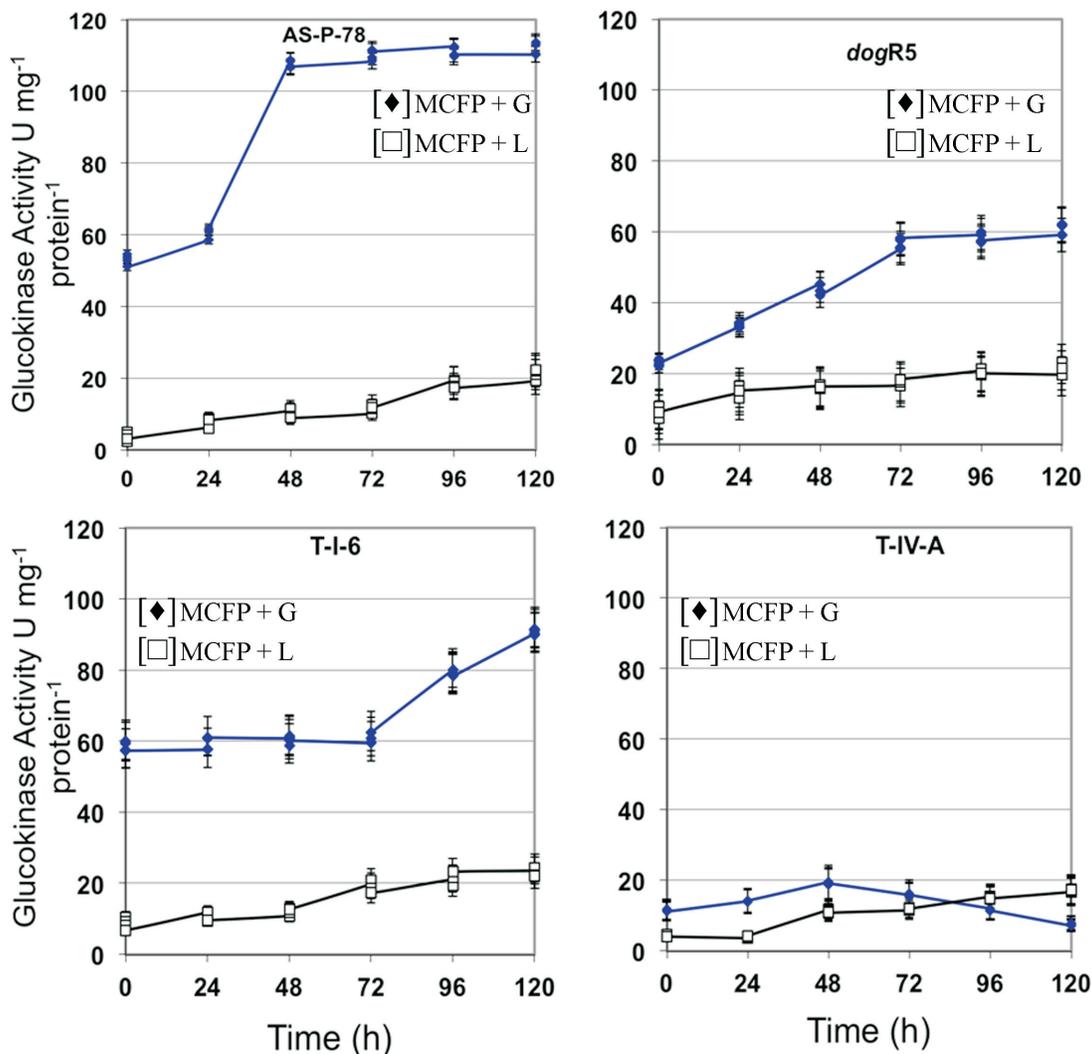


Figure 3 - Glucokinase activity in mycelium of strains AS-P-78, *dogR5*, and transformants T-I-6 and T-IV-A taken from submerged cultures in MCFP+G and MCFP+L media. Data correspond to the average of two repetitions from two different samples. Vertical bars represent standard deviations. Values showed significant difference ($p < 0.05$), except T-IV-A.

h of cultivation a β -galactosidase activity in glucose medium of approximately 50% the activity in lactose medium.

When all strains were grown on Czapek minimal medium with lactose as sole carbon source and supplemented with 3 mM D-2-deoxyglucose (D-2-DOG) the results correlated with the β -galactosidase present in each strain. Strain AS-P-78 does not grow in this condition, on having its β -galactosidase activity fully repressed by glucose, whereas strain *dogR5* forms compact, well developed colonies, with a smaller diameter and less sporulated than when growing on Czapek-lactose without D-2-DOG. In contrast, transformants T-I-6 and T-IV-4 show a much weaker growth than *dogR5*, not forming a uniform dense colony but a dotted area with poorly developed mycelium patches (Figure 6). These results indicate that the *HXK2* gene is able to revert, at least partially, CCR of β -galactosidase in the transformants.

Discussion

The results obtained in the present work confirm those found by Barredo *et al.* (1988) regarding the partial glucokinase deficiency of strain *dogR* and its deregulation in the glucose repression of penicillin biosynthesis. The data we have obtained in the study about glucokinase and hexokinase activities show that *P. chrysogenum* AS-P-78 and its derivative *dogR5* lack a functional hexokinase enzyme, which is in accordance with the described lack of fructose- and extremely low galactose-phosphorylating activities in both strains reported by Barredo *et al.* (1988). Most of studied fungi have separated enzymes for hexokinase and glucokinase activities, as happens in *Aspergillus nidulans* (Flippin *et al.*, 2003), *Aspergillus niger* (Panneman *et al.*, 1996, Panneman *et al.*, 1998), *Botrytis cinerea* (Rui and Hahn, 2007) or *Hypocrea jecorina* (Hartl and Seiboth, 2005), however a single unique enzyme carry-

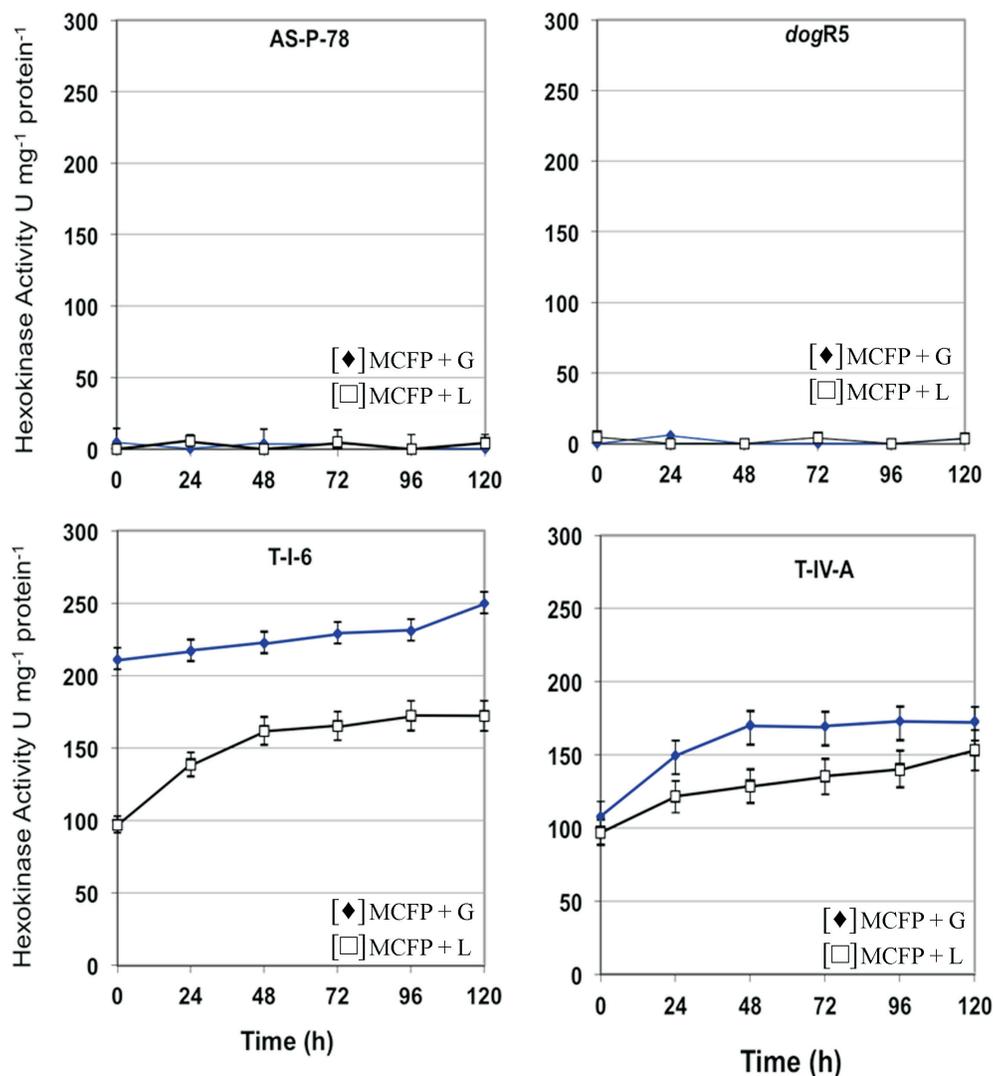


Figure 4 - Hexokinase activity in mycelium of strains AS-P-78, *dogR5*, and transformants T-I-6 and T-IV-A taken from submerged cultures in MCFP+G and MCFP+L media. Data correspond to the average of two repetitions from two different samples. Vertical bars represent standard deviations. Values of T-I-6 and T-IV-A showed significant difference ($p < 0.05$).

ing out both activities has been reported in *Kluyveromyces lactis*, the hexokinase KIHxk1 (Bär *et al.*, 2003). Recently a novel *K. lactis* enzyme has been described with glucokinase activity, but with a very low specific activity of glucose phosphorylation (2.5%) as compared to KIHxk1 (Kettner *et al.*, 2007).

Evidence presented in this work clearly suggests that an enzyme with glucokinase activity is involved in glucose regulation of penicillin biosynthesis and β -galactosidase in *P. chrysogenum*. The mutation in strain *dogR5* results in the loss of regulatory activity and part of the catalytic activity. Upon transformation with the *HXK2* gene the regulatory activity is recovered, but this activity is not linked to the catalytic glucokinase activity, which resulted altered in different ways in the transformants (higher in T-I-6 and lower in T-IV-A as compared to the parental *dogR5* strain). This

is not an unexpected result taking into account that catalytic and regulatory activities of hexokinases have been reported to be separated in yeasts (Hohmann *et al.*, 1999, Rodríguez *et al.*, 2001) and in plants (Moore *et al.*, 2003). Hexokinase activity, absent in strains AS-P-78 and *dogR5*, was also acquired by the transformants, demonstrating the functionality of the *HXK2* gene in the transformants. Hexokinase activity is higher in transformant T-I-6, the one with a stronger CCR, which might reflect a better expression of the *HXK2* gene. The simultaneous recovery of glucose regulation of penicillin biosynthesis and CCR upon transformation with the *HXK2* gene reinforces the hypothesis suggested by Barredo *et al.* (1988) of a common carbon regulatory pathway for primary and secondary metabolism in *Penicillium chrysogenum*.

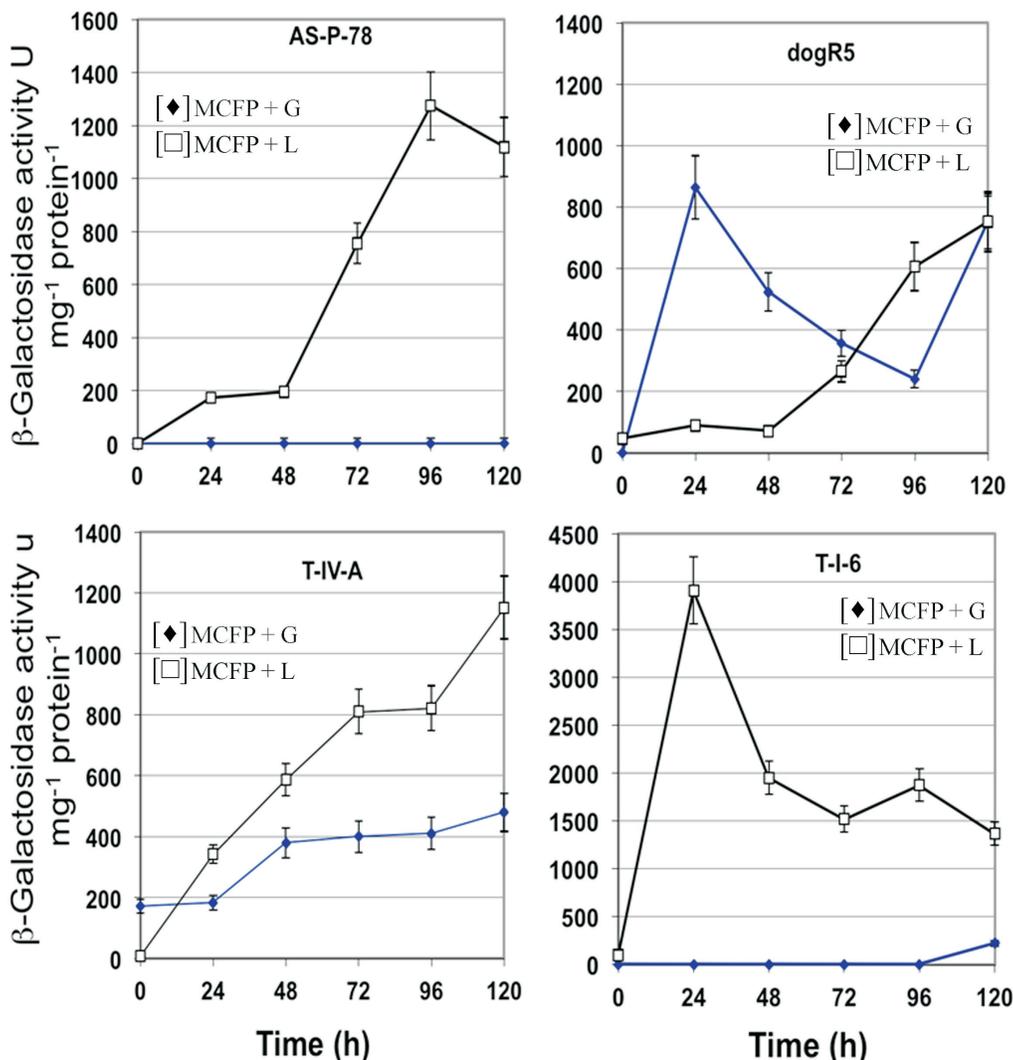


Figure 5 - β -galactosidase activity in mycelium of strains AS-P-78, *dogR5* and transformants T-I-6 and T-IV-A, taken from submerged cultures in MCFP+G and MCFP+L media. Data correspond to the average of two repetitions from two different samples. Vertical bars represent standard deviations.

Apart from the hexokinase Hxk2 of *S. cerevisiae*, other hexose phosphorylating enzymes have been implicated in CCR in some other fungi, as in the case of the double glucokinase/hexokinase KIHxk1 of *Kluyveromyces lactis* (Bär *et al.*, 2003), and both the hexokinase HxkA1 and the glucokinase GlkA4 from *Aspergillus nidulans* (Flippi *et al.*, 2003). In contrast, in *Botrytis cinerea*, neither glucokinase Glk1 nor hexokinase Hxk1 are involved in CCR (Rui and Hahn, 2007). Similarly to our results with strain *dogR5* of *P. chrysogenum*, *S. cerevisiae* HXK2 gene was also able to complement the CCR deficiency in a RAG5 (encoding KIHxk1) mutant of *Kluyveromyces lactis* (30). However, RAG5 failed to complement a HXK2 mutation in *S. cerevisiae*, which indicates that the molecular structure of hexokinases might play a discriminating role in protein-protein interactions mediating the CCR.

The precise role in CCR, of the enzyme responsible for the glucokinase activity in *Penicillium chrysogenum*, will be elucidated by analyzing the function of its encoding gene. The recently found evidence, that CreA represses expression of penicillin genes when *P. chrysogenum* is grown with glucose as carbon source (Fierro *et al.*, unpublished results), opens an interesting field for future research to investigate if CreA is regulated by nuclear location and if the glucokinase, or the Hxk2 hexokinase in the transformants, plays a role in this process, in a similar fashion as the interaction between Hxk2 and Mig1 in yeasts (Ahuatzi *et al.*, 2007). *Sclerotinia sclerotiorum* Cre1 is regulated by nuclear location of the protein in medium with glucose (Vautard-Mey *et al.*, 1999), although nuclear location and repressor activity of Cre1 seem to be separate functions in this fungus (Vautard-Mey *et al.*, 2000).

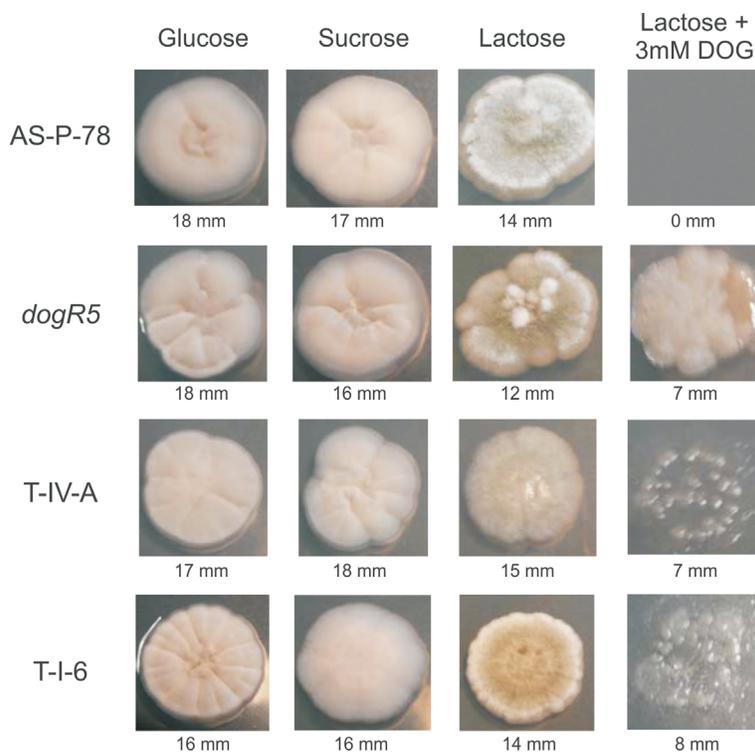


Figure 6 - Colonies from strains AS-P-78, *dogR5* and transformants T-I-6 and T-IV-A after nine days of growth on Czapek minimal medium with different carbon sources at a concentration of 3% (w/v) each. 3 mM D-2-deoxyglucose was added to the lactose-containing Czapek medium of the colonies shown on the right column. Images were taken with a Panasonic Lumix Model DMC-TZ3.

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