Transcription of the *pcbAB*, *pcbC* and *penDE* genes of *Penicillium chrysogenum* AS-P-78 is repressed by glucose and the repression is not reversed by alkaline pHs

Santiago Gutiérrez,^{1,2} Ana T. Marcos,² Javier Casqueiro,²
Katarina Kosalková,¹ Francisco J. Fernández,² Javier Velasco¹
and Juan F. Martín^{1,2}

Author for correspondence: Juan F. Martín. Tel: +34 987 291505. Fax: +34 987 291506.
e-mail: degjmm@unileon.es

¹ Area of Microbiology,
Faculty of Biology,
University of León, 24071
León, Spain

² Institute of Biotechnology,
INBIOTEC, Parque
Científico de León, Avda
del Real 1, 24006 León,
Spain

Glucose repressed transcription of the penicillin biosynthesis genes *pcbAB*, *pcbC* and *penDE* when added at inoculation time to cultures of *Penicillium chrysogenum* AS-P-78 but it had little repressive effect when added at 12 h and no effect when added at 24 or 36 h. A slight increase in the expression of *pcbC* and *penDE* (and to a smaller extent of *pcbAB*) was observed in glucose-grown cultures at pH 6.8, 7.4 and 8.0 as compared with pH 6.2, but alkaline pHs did not override the strong repression exerted by glucose. Transcription of the actin gene used as control was not significantly affected by glucose or alkaline pHs. Repression by glucose of the three penicillin biosynthetic genes was also observed using the *lacZ* reporter gene coupled to each of the three promoters in monocopy transformants with the constructions integrated at the *pyrG* locus. Glucose repression of the three genes encoding enzymes of penicillin biosynthesis therefore appears to be exerted by a regulatory mechanism independent from pH regulation.

Keywords: carbon catabolite regulation, pH effect, transcription, penicillin genes

INTRODUCTION

Antibiotics and other secondary metabolites are synthesized in response to nutritional stress, e.g. limitation of phosphate or of easily assimilable carbon and nitrogen sources (Martín & Demain, 1980; Martín & Liras, 1989). Secondary metabolites, accumulated in response to nutrient starvation, may serve as biochemical signals that trigger differentiation or as microbial antagonists that inhibit the growth of competing microorganisms (Chater & Bibb, 1997).

Penicillin biosynthesis in *Penicillium chrysogenum* is well known from the biochemical and genetic points of view (Aharonowitz *et al.*, 1992; Martín *et al.*, 1991; Martín, 1998). The three genes *pcbAB*, *pcbC* and *penDE* of *P. chrysogenum* involved in the conversion of the precursor amino acids into penicillin have been cloned (Carr *et al.*, 1986; Barredo *et al.*, 1989a, b; Díez *et al.*, 1990; Smith *et al.*, 1990; Tobin *et al.*, 1990) and they have been shown to be located in chromosome I (Fierro *et al.*, 1993) in a region that is easily amplified or deleted (Fierro *et al.*, 1996). Each of these three genes is

transcribed separately from an independent promoter; *pcbAB* and *pcbC* are transcribed divergently from promoters located in a 1.013 bp bidirectional promoter region (Díez *et al.*, 1990).

Penicillin biosynthesis in *P. chrysogenum* is strongly regulated by glucose. High glucose concentrations prevent formation of δ (L- α -aminoadipyl)-L-cysteinyl-D-valine (the first intermediate of the pathway), and depress isopenicillin N synthase and (only to a low extent) acyl-CoA:isopenicillin N acyltransferase (Revilla *et al.*, 1986). Penicillin biosynthesis is also regulated negatively by glucose in *Aspergillus nidulans* and the transcription of one of the biosynthetic genes (*ipnA*, homologous to *pcbC*) is known to be repressed in this fungus (Espeso & Peñalva, 1992). Espeso *et al.* (1993) have shown that transcription of the *A. nidulans* gene encoding the isopenicillin N synthase is under the control of the pH regulatory system mediated by the PacC regulatory protein and that external alkaline pH overrides carbon regulation. However, these authors proposed that carbon source regulation of the isopenicillin N synthase gene is mediated by a mechanism

independent of pH regulation in *A. nidulans*. The effect of pH on regulation of penicillin biosynthesis was also observed in *P. chrysogenum* (Chu *et al.*, 1997). Suárez & Peñalva (1996) recently proposed that there are differences between *Penicillium* and *Aspergillus* in carbon catabolite regulation of the penicillin pathway. Transcription of the *pcbC* gene has been studied by Kolar *et al.* (1991) and Feng *et al.* (1994); these authors reported the presence of glucose repression, but no detailed transcriptional analysis and transcription regulation studies of the *pcbAB* and *penDE* genes of *P. chrysogenum* have been carried out. Since we had available the three promoters of the penicillin biosynthetic genes, it was of interest to study if glucose control is exerted on the three penicillin biosynthetic genes or only on the *pcbC* gene, and if alkaline pH might reverse the repression exerted by glucose. Using Northern analysis and constructions in which the three promoters were coupled to the *lacZ* reporter gene in monocopy transformants, we have observed that an increase in the extracellular pH does not reverse carbon catabolite regulation, supporting the conclusion that the two regulatory phenomena take place by independent mechanisms.

METHODS

RNA from glucose-supplemented or unsupplemented cells.

P. chrysogenum AS-P-78, a high-penicillin-producing strain with the same degree of glucose regulation as the wild-type strain NRRL 1951, was used for most of the experiments. The low-producer strain Wis 54-1255 (Revilla *et al.*, 1984, 1986), was used to create the monocopy transformants (see Results). The latter strain was not selected for improved production in medium containing glucose.

Cultures of *P. chrysogenum* AS-P-78 were grown in Pharmamedia-lactose-phenylacetate medium (PLP) containing, per litre, Pharmamedia (Traders Protein, Forth Worth, TX, USA) 20 g, lactose 50 g, phenylacetic acid (40%) 10 ml, ammonium sulfate 4 g, calcium carbonate 5 g; pH 6.1. One hundred millilitre volumes of PLP medium in 500 ml flasks were inoculated with 7 ml of a seed culture grown for 36 h in CSYE medium containing, per litre, corn-steep solids 20 g, sucrose 20 g, yeast extract 5 g, CaCO_3 5 g; pH 5.7. Cultures in PLP medium were supplemented with 30 g glucose l^{-1} (166 mM) at 0 (inoculation time) 12, 24 and 36 h.

Total RNAs were extracted from each flask as described previously (Gutiérrez *et al.*, 1991), resolved in denaturing agarose formaldehyde gels (Ausubel *et al.*, 1987) and hybridized with probes internal to *pcbAB* (9.5 kb *Sall*-*EcoRI* fragment), *pcbC* (1.0 kb *NcoI* fragment) and *penDE* (1.3 kb *XbaI*-*XhoI* fragment). Control hybridizations were carried out with a probe internal to the actin gene (0.83 kb *NcoI*-*KpnI* fragment). Hybridization and washing conditions were as described previously (Gutiérrez *et al.*, 1991).

Reversion of glucose catabolite regulation by alkaline pHs. *P. chrysogenum* cells, grown at 25 °C for 16 h in Pharmamedia-phenylacetic medium with lactose (30 g l^{-1}) (PLP) or glucose (30 g l^{-1}) (PGP) as carbon source, were collected, washed and suspended in the same medium (without carbonate), buffered with 100 mM MES/100 mM monosodium phosphate and adjusted to pH 6.2, 6.8, 7.4 and 8.0 with 10 M NaOH. All

flasks were incubated at 25 °C for 6 h to determine the rate of penicillin production. Total RNAs were extracted and hybridizations (with probes internal to *pcbAB*, *pcbC*, *penDE* and β -actin) performed as described above.

Cell-free extracts and β -galactosidase activity. Mycelia from cultures grown in PLP and PGP media as indicated above were collected by filtration through Nylal filters, washed first with 0.9% NaCl and then with 50 mM sodium phosphate buffer pH 7.0, 1 mM MgCl_2 and 20 μM PMSF. After drying the mycelia on filter paper, they were frozen with liquid nitrogen and ground to a powder in a mortar at low temperature. Proteins were dissolved in 50 mM sodium phosphate buffer pH 7.0/1 mM EDTA/20 μM PMSF and clarified by centrifugation at 4000 g for 10 min at 4 °C. β -Galactosidase activity was quantified using ONPG as substrate (Miller, 1972).

RESULTS

Growth kinetics and levels of the *pcbAB*, *pcbC* and *penDE* transcripts

Rapid growth occurred in control lactose-grown cultures of *P. chrysogenum* AS-P-78 during the first 24 h, and then a moderate growth rate was maintained from 24 to 72 h. A very small increase in the mycelial dry weight occurred after 72 h (Fig. 1a). Under these conditions penicillin biosynthesis started at 24 h and proceeded linearly until 96 h.

The onset of transcription of the *pcbAB* gene in lactose-grown cultures of *P. chrysogenum* AS-P-78 in PLP medium occurred early (about 50% of the transcript was already formed at 24 h); the transcript levels of *pcbAB* increased significantly from 24 to 48 h and then reached a constant steady-state concentration (not shown). The transcript levels of the second gene of the pathway (*pcbC*) peaked at 48 h and then decreased slightly whereas the levels of transcript of the third gene (*penDE*) were low at 24 h and increased continuously up to 96 h. These results indicate that transcription of the *penDE* gene follows that of the *pcbAB* and *pcbC* genes, which is in agreement with the late formation of isopenicillin N acyltransferase (Alvarez *et al.*, 1987) and with the time of the penicillin production phase (from 24 to 96 h).

Transcription of *pcbAB*, *pcbC* and *penDE* with glucose or lactose as carbon source

Glucose (30 g l^{-1}) strongly reduced penicillin biosynthesis in *P. chrysogenum* AS-P-78 when added at inoculation time or at 12 h; its effect was smaller when added at 24 or 48 h (Fig. 1b–e). Glucose repressed formation of the *pcbAB*, *pcbC* and *penDE* transcripts particularly when added at inoculation time (0 h) and, to a lower extent, when added at 12 h of incubation (Fig. 2). A clear repressive effect was observed in the steady-state transcript levels of 72 h cultures when the glucose was added at 0 h but there was little or no repression when glucose was added at 12, 24 or 36 h (Fig. 2). The transcript level of the actin gene (used as control) was

Fig. 1. Effect of addition of glucose (30 g l^{-1}) at the time indicated by vertical arrows on growth ($\blacktriangle$), penicillin biosynthesis ($\bullet$) and pH of the cultures ($\circ$) for *P. chrysogenum* AS-P-78. $\triangle$, Residual glucose in the culture medium. The data points are the means of three determinations. (a) Control without glucose addition [lactose (50 g l^{-1}) was used as carbon source]; (b, c, d, e) glucose added at 0, 12, 24 and 48 h, respectively. The effect of glucose addition at 36 h (not shown) was similar to that observed with addition at 48 h.

not altered in glucose-supplemented cultures as compared to control cultures with lactose. No detectable levels of the *pcbAB* (not shown) and *penDE* transcripts and only a small level of *pcbC* transcript were observed at 72 h when glucose was added at inoculation time.

Since the steady-state levels of transcripts of the *pcbAB*, *pcbC* and particularly the *penDE* genes in lactose-grown cultures were still increasing at 12 and 24 h of growth, and the cultures showed a good growth rate at this time,

the repressing effect could be observed when glucose was added at 12 and even at 24 h after inoculation for the *penDE* gene.

Alkaline pHs do not override the glucose effect on transcription of *pcbAB*, *pcbC* and *penDE*

Northern analysis showed (Fig. 3a, b, c) that glucose strongly repressed expression of *pcbAB*, *pcbC* and *penDE* but the repression was not overridden by

Fig. 2. Steady-state levels of *pcbC* and *penDE* transcripts in cultures of *P. chrysogenum* AS-P-78 in PLP medium (lane 1) or in PLP medium supplemented with glucose (30 g l^{-1}) at 0, 12, 24 and 36 h (lanes 2, 3, 4 and 5, respectively). All RNAs were extracted from 72 h cultures. Five micrograms of each RNA sample was resolved in formaldehyde-agarose gel, blotted and hybridized with probes internal to the *pcbC*, *penDE* and actin genes, respectively (see text). The results obtained with RNAs extracted from 48 h cultures were similar.

Fig. 3. (a, b) Steady-state levels of *pcbAB*, *pcbC*, *penDE* and actin gene transcripts in cells of *P. chrysogenum* AS-P-78 suspended in (a) lactose-containing (PLP) or (b) glucose-containing (PGP) media adjusted to different pHs and incubated for 6 h at 25 °C. 1, RNA from unbuffered cultures; 2, pH 6.2; 3, pH 6.8; 4, pH 7.4; 5, pH 8.0. The panels in (b) have been overexposed (14 d) in order to observe the hybridizing signals, while those in (a) show a standard exposure (24 h). (c) Instant Imager quantification of the hybridizing bands. (d) Penicillin production by *P. chrysogenum* grown at different pHs as indicated for (a) and (b).

incubating the glucose-grown cells at neutral to alkaline pHs. The three transcripts were barely visible in glucose-grown cells and the films had to be overexposed for 2

weeks in order to show weak hybridization signals (Fig. 3), whereas they were clearly observed in lactose-grown cells exposed for only 1 d. When the intensity of the

Fig. 4. (a) Hybridization of total DNAs, digested with *Xho*I, from monocopy transformants obtained with plasmids pZ2bAB (lane 1), pZ2bC (lane 2) and pZ3bDE (lane 3) using a 370 bp *Sall*-*Eco*RI fragment corresponding to the *P. chrysogenum* *pyrG* gene as probe. Lane 4 shows one multicopy transformant with an additional 3.5 kb band indicating that other copies of the plasmid are integrated in the genome. (b) Scheme of the recombination process that occurs in the transformants of *P. chrysogenum* *npe10 pyrG3* with plasmids pZ2bAB, pZ2bC and pZ3bDE to obtain monocopy transformants. The sizes of the hybridization bands obtained with *pyrG* probe are also shown.

hybridizing band in Northern analysis was quantified, the highest signal was obtained in lactose-grown cultures maintained at pH 6.8, similar to the expression levels observed in unbuffered cultures grown in lactose.

A slight increase in the expression of *pcbC*, and to a lesser extent of *pcbAB* and *penDE*, was observed in glucose-grown cultures at pHs 6.8, 7.4 and 8.0 as compared to lower pHs, but expression of the three genes at the different pHs in glucose-containing medium was much lower than in lactose-containing medium (Fig. 3). At pH 5.0 a chemical degradation of all RNAs was observed.

The penicillin production in this experiment (Fig. 3d) was optimal in unbuffered lactose-grown cells and also in lactose-grown cells suspended at pH 6.8, but there was no significant penicillin production in glucose-grown cells suspended at alkaline pHs.

Reporter vectors with *lacZ*: gene targeting and isolation of monocopy integrants

Plasmids pZ2bAB, pZ2bC and pZ3bDE, containing, respectively, the promoters of the *pcbAB*, *pcbC* and *penDE* genes coupled to the *lacZ* gene, were constructed. Monocopy transformants of *P. chrysogenum* Wis 54-1255 (*npe10 pyrG*) with the plasmid integrated at the

pyrG locus were selected since *pyrG* recombination (single crossing-over) allowed growth in minimal medium due to complementation of the *pyrG*^{*} mutation. As shown in Fig. 4, monocopy transformants with pZ2bAB and pZ2bC showed in all cases two *Xho*I fragments of 8.5 kb and 4.0 kb, and monocopy transformants with pZ3bDE showed *Xho*I fragments of 10.1 kb and 4.0 kb as expected (Fig. 4).

Effect of carbon source and pH on the *pcbAB*, *pcbC* and *penDE* promoters coupled to the reporter *lacZ* gene

As shown in Table 1, transformants carrying single copies of the constructions with the *lacZ* gene coupled to the *pcbAB*, *pcbC* and *penDE* promoters integrated at the *pyrG* locus (Fig. 4b), showed a much higher β -galactosidase activity in cultures grown in lactose than in cultures grown in glucose. *penDE* is expressed very poorly in monocopy transformants when integrated in the *pyrG* locus.

The β -galactosidase activities observed at pH 8.0 (Table 1) clearly indicated that alkaline pHs do not reverse the glucose effect on expression of *lacZ* from the *pcbAB*, *pcbC* and *penDE* promoters in *P. chrysogenum*, confirming the results obtained with the Northern hybridization experiments.

Table 1. β -Galactosidase activity of monocopy transformants carrying the *pcbAB*, *pcbC* and *penDE* promoters coupled to *lacZ*

β -Galactosidase activity was determined in monocopy transformants of *P. chrysogenum* Wis 54-1255 (*npe10 pyrG3*) with the plasmids pZ2b (without insert), pZ2bAB (*pcbAB* promoter), pZ2bC (*pcbC* promoter) and pZ3bDE (*penDE* promoter) using either glucose or lactose as carbon source. The pH was 8.0 in each case. The results are means of two determinations.

Promoter	Carbon source	β -Galactosidase activity [units (mg protein) ⁻¹]
None	Lactose	12.66
	Glucose	0.20
<i>pcbAB</i>	Lactose	122.54
	Glucose	9.40
<i>pcbC</i>	Lactose	260.23
	Glucose	9.33
<i>penDE</i>	Lactose	14.15
	Glucose	0.31

DISCUSSION

Glucose regulation of penicillin biosynthesis in different strains of *P. chrysogenum* has been known for many years (Revilla *et al.*, 1984, 1986). Glucose was shown to reduce formation of the ACV tripeptide and to repress *in vivo* the isopenicillin N synthase and, to a much smaller extent, the acyl-CoA:6-aminopenicillanic acid acyl-transferase activity. The lack of a simple assay for ACV synthetase at that time prevented further studies on glucose regulation of the formation of this enzyme in *P. chrysogenum*. Zhang & Demain (1989) showed that glucose repression of cephalosporin biosynthesis in *Acremonium chrysogenum* acted on the ACV synthetase level.

As shown in this article, the steady-state levels of the *pcbAB*, *pcbC* and *penDE* transcripts are drastically reduced in cultures supplemented with glucose at inoculation time. Although the drastic decrease in the steady-state levels of the *pcbAB*, *pcbC* and *penDE* transcripts could also be due to increased degradation of the transcripts in glucose-supplemented cells, a direct effect of glucose on preventing transcription is supported by the severe reduction in β -galactosidase activity observed in monocopy constructions (integrated at the *pyrG* locus) in which the reporter *lacZ* gene was coupled to each of the three promoters. A similar glucose repression of the *ipnA* (= *pcbC*) gene of *A. nidulans* is known (Brakhage *et al.*, 1992; Pérez-Esteban *et al.*, 1993) but Brakhage and coworkers reported that expression of the *pcbAB* gene was not repressed in *A. nidulans*. Although there are differences in the organization and particularly in the amplification of the penicillin genes in *P. chrysogenum* and *A. nidulans* (Fierro *et al.*, 1993, 1995) the basic regulatory mech-

anisms of carbon and nitrogen regulation were believed to be similar in both fungi (Kolar *et al.*, 1991; Feng *et al.*, 1994). However, Suárez & Peñalva (1996) proposed that the mechanisms of control by pH and carbon catabolite regulation are different in *P. chrysogenum* and *A. nidulans*. Our results fully support that proposal.

The steady-state levels of transcripts of the *pcbAB* and *penDE* genes kept increasing until 72–96 h; the *pcbC* transcript reached a peak at 48 h and then levelled off under standard culture conditions; however, little or no glucose repression was observed when glucose was added at 12, 24 or 36 h, when mRNA synthesis from the three penicillin genes was still very active. These results are consistent with the hypothesis that glucose (when added early to the culture) represses or modifies a carbon regulatory protein that interacts with the *pcbAB*, *pcbC* and *penDE* promoters. In *A. nidulans* carbon catabolite repression of primary metabolism gene expression is mediated by the transcriptional regulatory protein encoded by the *creA* gene (Arst & MacDonald, 1975; Bailey & Arst, 1975; Dowzer & Kelly, 1991; Kulmburg *et al.*, 1993). However, in *A. nidulans* all tested *creA* mutants affected the *ipnA* mRNA levels only slightly (Espeso & Peñalva, 1992). At this time it is unclear whether CreA is involved in the glucose control of penicillin biosynthesis in *P. chrysogenum*.

We have shown previously that a 2-deoxyglucose-resistant mutant of *P. chrysogenum*, which has constitutive endogenous β -galactosidase, is derepressed in penicillin biosynthesis when grown with high levels of glucose (Barredo *et al.*, 1988). This mutant, which is deficient in glucokinase activity, might be altered in the DNA-binding protein that mediates carbon catabolite regulation.

The carbon regulatory protein appears to be formed (or modified) in a short 'time window' following inoculation of the penicillin fermentations since the repressive effect is clearly lower when glucose is added after 12 h of incubation.

Derepression of *pcbAB*, *pcbC* and *penDE* was rapidly observed following glucose depletion. This observation explains why the repressive effect of glucose on isopenicillin N acyltransferase (the last enzyme of the penicillin pathway) is generally very weak (Barredo *et al.*, 1988; Revilla *et al.*, 1986). Isopenicillin N acyltransferase is formed late in the culture (Alvarez *et al.*, 1987), when most of the glucose has already been utilized. However, as shown in this work, transcription of *penDE* is sensitive to glucose during the early growth phase of the culture.

In addition, a pH regulatory circuit is known in *A. nidulans* which controls extracellular enzymes, permeases and other cellular processes (Caddick *et al.*, 1986). The pH control is mediated by the PacC regulatory protein (Tilburn *et al.*, 1995). The extracellular pH also affects penicillin production as shown in this article, in agreement with the results of Shah *et al.* (1991) for *A. nidulans* and Chu *et al.* (1997) for *P. chrysogenum*. Espeso *et al.* (1993) proposed that external alkaline pH

in *A. nidulans* overrides sucrose regulation of *ipnA*. In *P. chrysogenum* our results show that alkaline extracellular pH produces a small stimulation of transcription of *pcbAB*, *pcbC* and *penDE*, but it does not override the strong catabolite regulation exerted by glucose. It is likely that there are differences in the fine control of penicillin gene expression in *P. chrysogenum* AS-P-78 and *A. nidulans* (Suárez & Peñalva, 1996), thus explaining the much higher expression of the penicillin genes in *P. chrysogenum* than in *A. nidulans*.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from Antibioticos SpA (Milan, Italy). K. Kosalková received a fellowship from the Ministerio de Asuntos Exteriores (Madrid, Spain), and F. J. Fernández and J. Velasco from the University of León. We thank Daniel Ramón (Valencia, Spain) for providing a probe internal to the actin gene of *A. nidulans*, and Cees A. M. J. J. van den Hondel (TNO, The Netherlands) for providing plasmids pAN923-42A and pAN923-43A.

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Received 2 June 1998; revised 5 November 1998; accepted 13 November 1998.