III. REPRESSION OF PHENYLACETIC ACID TRANSPORT SYSTEM IN *PENICILLIUM CHRYSOGENUM* WIS 54-1255 BY FREE AMINO ACIDS AND AMMONIUM SALTS

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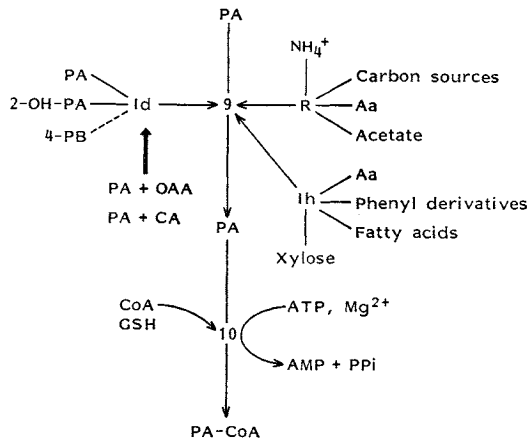
The phenylacetic acid (PA) transport system in *Penicillium chrysogenum* is an inducible-system (see FERNÁNDEZ-CAÑÓN *et al.*; preceding papers) which is repressed by free amino acids when these molecules are added to the complex fermentation broths at the induction time. L-Tyrosine, L-α-aminoadipic acid, L-tryptophan, L-phenylalanine and L-methionine are the molecules that cause the greatest delay in induction. The addition of Krebs-cycle intermediates to the complex fermentation broth did not affect the rate of induction with the exception of oxalacetic acid and citric acid which strongly increased it. Ammonium salts and acetate also repressed the biosynthesis of the enzymes involved in the PA uptake.

The effects of different compounds on the biosynthesis of secondary metabolites[1] and more particularly on the production of antibiotics are well known[1~10]. Variations in the nature and concentration of the nutrients further affect the final titers and recovery of these molecules[1~3].

The benzylpenicillin biosynthesis as in the case of many other antibiotics is repressed by rapidly metabolized carbon[11~20] and nitrogen[4,5] sources although the precise molecular mechanisms involved in this regulation remain to be elucidated.

It has been reported that δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACV) and isopenicillin N synthase are under carbon catabolite regulation in *Penicillium chrysogenum*[20]; however, these two enzyme are common to the biosynthesis of all β-lactam-containing molecules produced by this fungus and hence are not specific for benzylpenicillin biosynthesis. In the preceding papers, we have reported that the phenylacetic acid (PA) transport system (enzymatic step No. 9) is an inducible system which is under catabolite regulatory control (see Fig. 1). We have also reported that L-lysine, the end product of one of the two branches of the benzylpenicillin biosynthetic pathway, represses the PA transport system when it is added to the fermentation broth at induction time. However, it is still not clear whether this regulation is

Fig. 1. Modulation of the PA transport system (enzymatic step No. 9).



Id: Induction, R: repression, Ih: inhibition, Id and a thick arrow: quick induction, 2-OH-PA: 2-hydroxyphenylacetic acid, 4-PB: 4-phenylbutyric acid, Aa: amino acids, OAA: oxalacetic acid, CA: citric acid, 9: PA transport system, 10: phenylacetyl coenzyme A ligase, dashed line: indirect induction.

specifically caused by this amino acid (as in the case of homocitrate synthase)[21~24] or whether lysine acts in carbon catabolite repression, since *P. chrysogenum* is able to use the carbon skeleton of this molecule as energy source. In order to further clarify this point, the effect caused by other amino acids and their catabolites (Krebs-cycle intermediates), nitrogen and phosphorous sources on the induction of PA transport system has now been tested.

Experimental

Chemicals

All the products employed were of analytical quality.

Microorganisms

P. chrysogenum Wis 54-1255 (ATCC 28089) was obtained from the American Type Culture Collection. The strain was maintained in the lyophilized state[25,26].

Culture Media and Growth Conditions

P. chrysogenum Wis 54-1255 was maintained and cultured as described (see the preceding paper).

Induction Experiment

To establish the effect of different molecules on the induction of the PA transport system, *P. chrysogenum* was grown in the medium described above under identical physical conditions but without PA. After 60 hours of growth (or the time required), PA (0.1% w/v) or PA (0.1% w/v) and different molecules were added to the flasks; the effector were added 1 hour before PA. The final concentrations of these compounds were adjusted keeping the nitrogen (for amino acids and ammonium salts) or the carbon (for Krebs-cycle intermediates) concentration constant and equivalent to the nitrogen or carbon present in 25 mM L-lysine. In the particular case of L-cysteine, the final concentration was lower (equivalent in nitrogen to 5 mM L-lysine) since high concentrations of this amino acid are toxic for the fungus. The induction of the PA transport system was followed at different times after adding PA (6, 12 and 24 hours).

Transport Experiments

Mycelia of *P. chrysogenum* grown for 60 hours and induced in the above media and conditions were harvested at different times (6, 12 and 24 hours) and treated as described in the preceding papers.

Ammonium Determinations

Ammonium was measured in the culture media using an Orion electrode (Cambridge, MA, U.S.A.) as previously described[27] or an enzymatic electrode (Hitachi 704 Autoanalyzer).

Enzymatic Assays

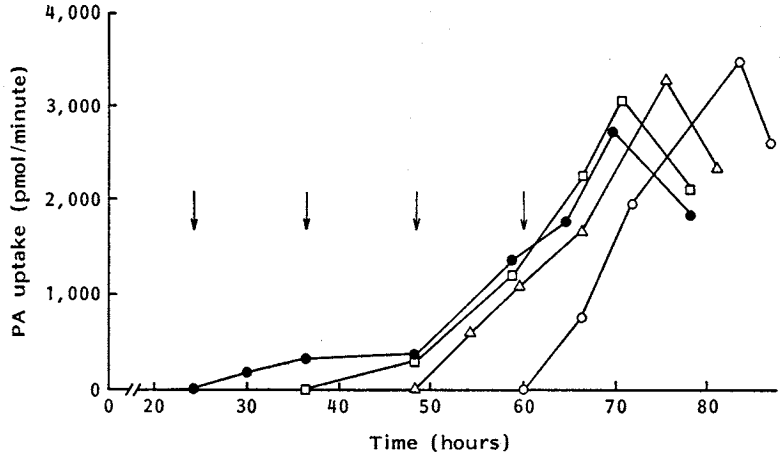
Phenylacetyl-CoA: 6-Aminopenicillanic acid (6-APA) acyltransferase, isopenicillin N synthase and isocitrate lyase were assayed as previously described[25,26,28,29].

Results and Discussion

The optimal induction time for the PA transport system in *P. chrysogenum* was studied. We found that the maximal transport of PA was achieved when PA was added to the broths after 60 hours of fungal growth. At other times, the rates of induction were somewhat lower and in all these cases the maximum uptake capacities were also reduced (Fig. 2).

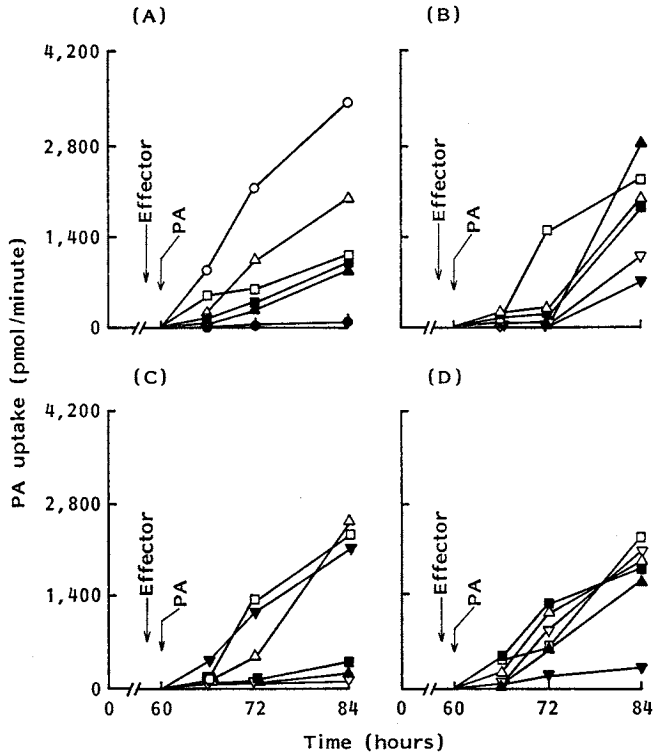
The effect of amino acids, Krebs-cycle intermediates, ammonium salts and phosphate on the induction of the PA transport system was studied. All the amino acids tested caused a notable delay in induction, L-tyrosine, L-α-aminoadipic acid, L-tryptophan, L-phenylalanine and L-methionine being the molecules that caused the great degree of repression (Fig. 3). The fact that D(−)-4-hydroxy-

Fig. 2. Induction of PA transport system in *Penicillium chrysogenum* Wis 54-1255 at different times of growth.



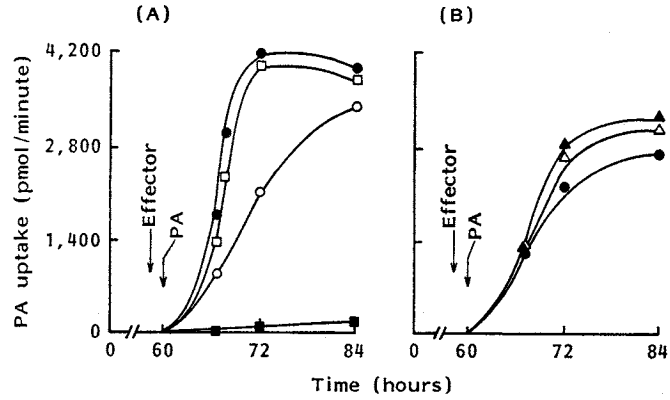
Arrows indicate the time at which PA (0.1% w/v) was added to the flasks (● 24 hours, □ 36 hours, △ 48 hours, ○ 60 hours).

Fig. 3. Effect of different amino acids on the induction of PA transport system in *Penicillium chrysogenum* Wis 54-1255.



(A) ○ Control (PA, 0.1% w/v added at 60 hours), □ + L-Asp, △ + L-Glu, ■ + L-Asn, ▲ + L-Gln, ● control (without PA).
(B) □ + L-Ala, △ + L-Leu, ■ + L-Ile, ▲ + L-Val, ▽ + L-Cys, ▼ + L-Met.
(C) □ + L-Ser, △ + L-Thr, ■ + L-Phe, ▲ + L-Trp, ▽ + L-Tyr, ▼ + 4-OH-phenylglycine.
(D) □ + Gly, △ + L-Arg, ■ + L-His, ▲ + Pro, ▽ + L-Lys, ▼ + α-aminoadipic acid

Fig. 4. Effect of Krebs-cycle intermediates on the induction of PA transport system in *Penicillium chrysogenum* Wis 54-1255.



(A) ○ Control (PA, 0.1% w/v added at 60 hours), ● + OAA, □ + CA, ■ control without PA.
(B) ● + α-ketoglutarate, △ + succinate, ▲ + malate.

phenylglycine (an amino acid analogue) (Figs. 3C and 3D) also repressed the PA uptake system suggests that the effect caused by amino acids is not due to an increase in protein synthesis (stopping the idiophase and restarting the trophophase) but rather to a different regulatory mode of control.

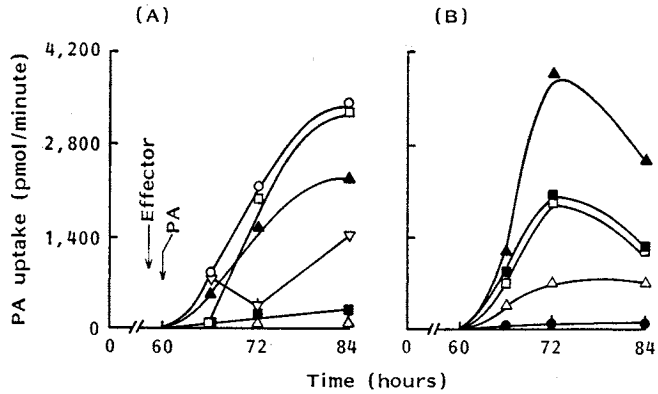
In order to establish whether the effect of amino acids is due to carbon[11~20] or nitrogen regulation[4,5], the effect of Krebs-cycle intermediates was also tested. These molecules are produced from catabolism of amino acids, sugars and fatty acids and therefore could cause a similar regulatory control. Fig. 4 shows that none has a significant influence of the PA uptake with the exception of oxalacetic acid (OAA) and citric acid (CA) that strongly stimulated the rate of its induction.

It could be speculated that these acids (OAA and CA) could increase the activities of some of the enzymes involved in the last step of benzylpenicillin biosynthesis and hence the uptake of PA. However, neither OAA nor CA are seen to affect ACV synthetase, isopenicillin N synthetase or acyltransferase suggesting that the stimulation above described is not related with the activity of these three enzymes (results not shown).

It is also possible that OAA and CA increased the rate of the PA uptake induction by blocking the synthesis of some compound that would play a negative role as a metabolic signal. CA, like ATP, controls the glycolytic pathway by inhibiting phosphofructokinase and as a result, acetyl-CoA is not produced efficiently[30]. Moreover, OAA is condensed with a molecule of acetyl-CoA to form CA[31] and, therefore, an excess of OAA might deprive the cell of acetyl-CoA. We have described (see above) that amino acids strongly repressed PA uptake system. Therefore, in the presence of nitrogen source (ammonium salts), OAA and α-ketoglutaric acid would be transformed into amino acids and hence the stimulation of PA uptake would not be expected. However, under our assay conditions (at 60 hours of growth) the mycelia of *P. chrysogenum* is under nitrogen-limiting conditions and amino acids are poorly synthesized even in the presence of exogenous supplies of Krebs-cycle intermediates. By this reasoning OAA and CA can even increase the uptake of PA.

Attempting to establish whether the hypothesis in which acetyl-CoA would play an regulatory role in carbon catabolite regulation of the PA transport system is correct, sodium acetate was added to the broth (59 hours). Fig. 5A shows that no PA transport was observed in the first 6 hours of

Fig. 5. Effect of different metabolites, ammonium salts and phosphate on the induction of PA transport system in *Penicillium chrysogenum* Wis 54-1255.



(A) ○ Control (PA, 0.1% w/v added at 60 hours), □ + acetate, ■ + NH_4 acetate, △ + acetate and itaconate (100 mM), ▲ + pyruvate, ▽ *idem* control but at 6 hours of induction 25 mM L-Lys was added to the flasks.

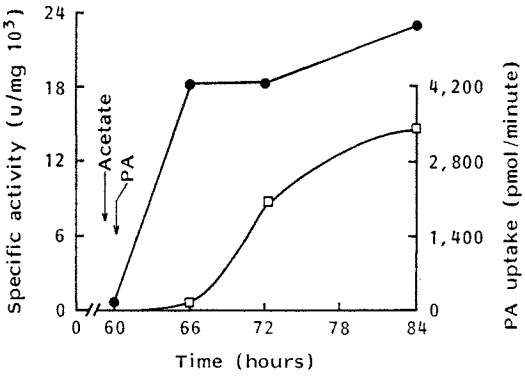
(B) ● Control without PA, □ + ammonium sulfate, ■ + ammonium chloride, △ + OAA and ammonium sulfate, ▲ + KH_2PO_4.

induction whereas later the PA uptake was rapidly induced (reaching similar levels as the controls in which only PA was supplied). These results suggest that during the first hours of induction, acetate is transformed in acetyl-CoA which strongly repressed the transport of PA. This negative effect will later be reversed by induction of the enzymes involved in the glyoxylic acid cycle which will exhaust the intracellular pool of acetyl-CoA.

Measurement of isocitrate lyase activity (a key enzyme in this cycle) at different induction times shows that the enzyme is not present in mycelia harvested at 60 hours and it was synthesized after adding acetate (Fig. 6). Accordingly, we propose that acetyl-CoA is efficiently synthesized after the addition of acetate and, under these conditions, the enzyme(s) involved in PA uptake would be repressed. Later, when the enzymes of the glyoxylic cycle are induced, the pool of acetyl-CoA would decreases and hence the PA uptake system would be induced. Furthermore, the addition of methylene succinic acid (itanoic acid, a potent inhibitor of isocitrate lyase[32]) together with acetate prevented the rapid induction of the PA transport system observed at 12 hours of incubation when only acetate was supplied (Fig. 5A). These results lend further support to the above hypothesis, suggesting that acetyl-CoA must be removed so that the enzyme(s) required for PA transport to be synthesized. These results agree with those reported by ROMANO and KORNBERG who demonstrated that in fungi acetyl-CoA regulated the activities of some of the enzymes involved in the transport of several carbohydrates[33].

It is clear that the regulation caused by amino acids in the induction of this uptake system is not due to the carbon skeleton provided by such molecules (since Krebs-cycle intermediates should also repress it) but rather to the presence of nitrogen atoms in their structures. It has been previously reported[4,5] that benzylpenicillin biosynthesis in *P. chrysogenum* is under nitrogen catabolite regulation. Accordingly, in order to establish whether the transport of PA is also repressed by other nitrogen-containing molecules, several ammonium salts (acetate, chloride and sulfate) were tested. Fig. 5 shows that the induction rates in the first hours were very similar to the controls, whereas later (12~

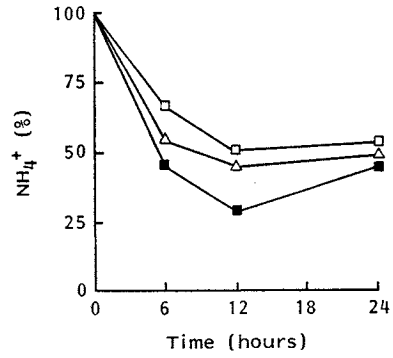
Fig. 6. Appearance of the isocitrate lyase activity after adding acetate (as effector) to the flasks in which PA transport system was induced.



● Isocitrate lyase activity, □ PA uptake when sodium acetate was added to the flasks at 59 hours. Isocitrate lyase activity is given as U per mg of protein (one U of enzyme is defined as the amount of enzyme which catalyzes the formation of 1 μmol of glyoxylate phenylhydrazone per minute).

Fig. 7. Consumption of ammonia by *Penicillium chrysogenum* Wis 54-1255 when different ammonium salts were tested as effectors on the induction of PA transport system.

□ NH_4Cl, ■ CH_3COONH_4, △ $(NH_4)_2SO_4$.



24 hours) the uptake of PA decreased. The repression pattern is different from that caused by amino acids suggesting that a high intracellular level of NH_4^+ must be attained before repression can take place. It is well known that NH_4^+ is easily transported across cell membranes (Fig. 7), so this pattern of repression cannot be explained merely in terms of the high levels of this cation inside the cells. We suggest that the absence of repression during the first hours of induction (6 hours) is due to the low intracellular pool of free amino acids. Later (after 6 hours) when the need of amino acids for protein synthesis has been satisfied, the pool would increase, causing a negative effect similar to that described above.

The fact that when oxalacetic acid and NH_4^+ were added to the broth very low rates of the induction were observed (Fig. 5B) indicates that amino acids can be efficiently synthesized. This result agrees with the decrease in PA uptake observed when 25 mM L-lysine was added to the broth after 6 hours of induction (Fig. 5A) (see preceding papers).

Ammonium acetate, also caused a strong repression in the induction of the PA uptake system. In this case, unlike sodium acetate, no induction was observed at any time, suggesting that during the first hours (before 6 hours of induction) PA uptake was repressed by acetate (acetyl-CoA) whereas later, when the uptake system is induced by the existence of a functional glyoxylic cycle that eliminates the excess of acetyl-CoA, the ammonium provided by the salt permits the synthesis of amino acids which, as above described, prevent the induction.

The supply of phosphate to the broth, unlike systems involved in the biosynthesis of other antibiotics[34,35], did not repress the induction of the PA uptake system but instead stimulated it (Fig. 5B). The presence of phosphate is thought to increase the intracellular pool of ATP and other molecules[36] that might provide the energy necessary for this active transport system.

Knowledge of the enzymatic steps under catabolite regulation as well as the precise mechanisms by which carbon, nitrogen or phosphorus control it, would be very important not only for the development of new strategies to achieve more efficient selection methods for overproducing mutants but also for improving the yields of industrial fermentations of antibiotics by using a more rational model

of operation, taking into account the metabolic requirements of the strains and avoiding the limiting steps that hinder the synthesis of the required molecule.

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