II. PHENYLACETIC ACID TRANSPORT SYSTEM IN *PENICILLIUM CHRYSOGENUM* WIS 54-1255: MOLECULAR SPECIFICITY OF ITS INDUCTION

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The phenylacetic acid (PA) transport system of *Penicillium chrysogenum* is induced by PA, 2-hydroxyphenylacetic and 4-phenylbutyric acids but not by benzoic, phenoxyacetic acid and phenylpropionic acids. Substitution in the aromatic moiety (3-hydroxyphenylacetic, 4-hydroxyphenylacetic acids), replacement of the aromatic moiety by other rings (thiophene-2-acetic acid, indole-3-acetic or indole-3-butyric acids) or the presence of an amino group in the α-position (2-aminophenylacetic acid) eliminates inducing activity. 2-Phenylbutyric acid dose not induce the PA transport system indicating that fatty acid-β-oxidation is needed to generate the authentic regulatory molecule (phenylacetyl-CoA) from 4-phenylbutyric acid. Furthermore, the uptake system synthesized in presence of PA, 2-hydroxyphenylacetic or 4-phenylbutyric acids is under carbon catabolic repression control and is also repressed by L-lysine suggesting that the three molecules induce in *P. chrysogenum* a single mechanism of transport.

Penicillium chrysogenum Wis 54-1255 produces isopenicillin N, 6-aminopenicillanic acid (6-APA) and different penicillins (benzyl, phenoxymethyl, DF, F and K). These molecules are synthesized either by removing the L-α-aminoadipic acid moiety present in the isopenicillin N molecule and then acylating the free 6-APA produced or by exchanging the α-aminoadipic acid moiety with other side chain precursors such as phenylacetic acid (PA) (G), phenoxyacetic acid (POA) (V), hexenoic (F), hexanoic (DF), octanoic (K) or *p*-hydroxyphenylacetic acids (X)[1,2]. These transfer reactions are brought about in two or at least three different enzymatic steps depending on the endogenous (intracellular) or exogenous origin of the side chain precursor molecule respectively. When the compounds are of endogenous origin (synthesized by *P. chrysogenum*), they are first activated to their CoA derivatives and later a different enzyme (acyl-CoA: 6-APA acyltransferase)[1] catalyzes either *N*-acylation of the penicillin nucleus (6-APA) or direct transacylation between the acyl-CoA and the α-aminoadipic acid moiety of the isopenicillin N molecule as described above. However, when the penicillin side chain precursor molecules cannot be synthesized in *P. chrysogenum*, they must be obtained exogenously. In the preceding paper we have characterized the PA uptake in *P. chrysogenum* and we established that it was a inducible system. In the present report we studied which molecules, besides PA, can act as inducers of this transport system.

Experimental

Chemicals

All the products used were of analytical quality.

Microorganism

P. chrysogenum Wis 54-1255 (ATCC 28089) was obtained from the American Type Culture Col-

lection. The strain was kept lyophilized.

Culture Media and Growth Conditions

P. chrysogenum Wis 54-1255 was maintained and cultured as described (see FERNÁNDEZ-CAÑÓN *et al.* in the preceding paper). *P. chrysogenum* was grown in 500-ml Erlenmeyer flasks containing 60 ml of liquid medium (corn-steep solids 30 g, lactose 30 g, PA 1 g, $CaCO_3$ 10 g and distilled water to 1 liter). Each flask was seeded with 1 ml of a spore suspension (2×10^8 spore/ml) and incubated at 25°C and 250 rpm for 49 hours. At this time glucose (2% w/v); L-lysine (50 mM) or sterile distilled water was added and 1 hour later the inducer molecule (at 0.1% w/v final concentration).

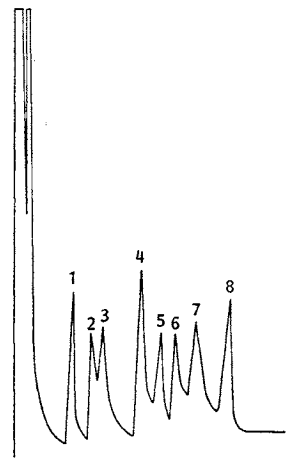
Transport Experiments

Mycelia of *P. chrysogenum* grown for 49 hours were induced in the above medium, harvested at different times (6, 12 and 24 hours) and washed four times with sterile distilled water. Aliquots of 35 mg of weight (about 10 mg of dry weight) were suspended in 25-ml Erlenmeyer flasks containing 1.4 ml of 0.06 M phosphate buffer, pH 6.5 and preincubated at 25°C for 5 minutes in a thermostatically controlled bath at 160 strokes/minute before adding PA (6.4 μM containing 1.4 μM of labeled PA). Incubations were carried out for 30 seconds, 10 ml of water was added and the suspension was rapidly filtered through Millipore filters (0.45 μm pore size). The filters were washed 3×10 ml of sterile distilled water. The radioactivity of the filters were measured as previously described[3]. PA uptake is given as pmol/minute.

Separation by Gas-liquid Chromatography of Different Phenyl Derivatives

Benzoic, 2-hydroxyphenylacetic, PA, 2-hydroxybenzoic, 3-phenylpropionic, 3-(2-hydroxyphenyl)-propionic, POA and 4-phenylbutyric acids can be completely separated using a GC (Varian 3300) equipped with a flame ionization detector and a fused silica megabore column (15 m×0.53 mm) (J and W Scientific Inc. California, U.S.A.). Oven temperature was kept at 120°C for 5 minutes and then programmed with 4°C/minute to 160°C. Nitrogen flow rate was 9 ml/minute. The solvent used for the different compounds was chloroform - methanol (2:1). Under these conditions a suitable and rapid method for the simultaneous determination of these aromatic molecules is achieved (see Fig. 1).

Fig. 1. GC obtained from different phenyl derivatives.



1: Benzoic acid, 2: 2-hydroxyphenylacetic acid, 3: PA, 4: 2-hydroxybenzoic acid, 5: 3-phenylpropionic acid, 6: 3-(2-hydroxyphenyl)propionic acid, 7: POA, 8: 4-phenylbutyric acid. Retention time were 2.81, 3.56, 4.02, 5.58, 6.33, 6.86, 7.70 and 9.06 minutes, respectively.

Identification of the Penicillin Produced by *P. chrysogenum* when 4-Phenylbutyric Acid was used as Side Chain Precursor

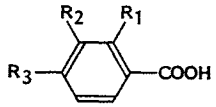
P. chrysogenum fermentations were carried out in the presence of phenylbutyric acid (0.1% w/v added at zero time). At 60 hours of growth, 2 liters of culture broths were collected and centrifuged at $18,000\times g$ for 10 minutes. The supernatant fluid was centrifuged again for 5 minutes and ammonium sulfate was added (final concentration, 45% w/v). Penicillins with no polar side chains present in the solution immediately began to precipitate[4,5]. The precipitate was separated by filtration through a No. 4 Pyrex glass-fiber filter and quickly extracted with butylacetate as previously described[6]. The rich butylacetate fraction was treated with ammonium 2-ethylhexanoate[1] and after drying *in vacuo* a powder was obtained. This was washed with dry acetone and dried in a oven (28°C overnight). The white solid obtained was hydrolyzed with 5 N HCl (5 ml) at 80°C for 10 hours (N_2 atmos-

phere)[1]. The resulting solution was vigorously extracted with diethyl ether (15 ml), the organic phase was separated, dried under reduced pressure and aliquots of the residual oil were analyzed by GC after dissolving them in chloroform - methanol (2 : 1) as described above.

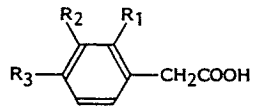
Results and Discussion

Of all the phenyl derivative compounds (or close structural molecules) tested (Fig. 2) only compounds PA (**5**), 2-hydroxyphenylacetic acid (**6**) and 4-phenylbutyric acid (**15**) were able to induce the

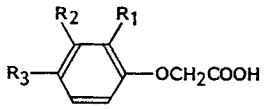
Fig. 2. Molecules tested as possible inducers of the PA transport system of *Penicillium chrysogenum* Wis 54-1255.



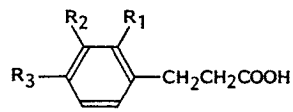
Benzoic acid (**1**) $R_1=R_2=R_3=H$
2-Hydroxybenzoic acid (**2**) $R_1=OH$ $R_2=R_3=H$
3-Hydroxybenzoic acid (**3**) $R_1=R_3=H$ $R_2=OH$
4-Hydroxybenzoic acid (**4**) $R_1=R_2=H$ $R_3=OH$



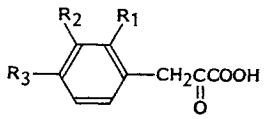
PA (**5**) $R_1=R_2=R_3=H$
2-Hydroxyphenylacetic acid (**6**) $R_1=OH$ $R_2=R_3=H$
3-Hydroxyphenylacetic acid (**7**) $R_1=R_3=H$ $R_2=OH$
4-Hydroxyphenylacetic acid (**8**) $R_1=R_2=H$ $R_3=OH$



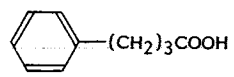
POA (**9**) $R_1=R_2=R_3=H$



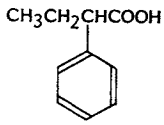
3-Phenylpropionic acid (**10**) $R_1=R_2=R_3=H$
3-(2-Hydroxyphenyl)-propionic acid (**11**) $R_1=OH$ $R_2=R_3=H$
3-(3-Hydroxyphenyl)-propionic acid (**12**) $R_1=R_3=H$ $R_2=OH$
3-(4-Hydroxyphenyl)-propionic acid (**13**) $R_1=R_2=H$ $R_3=OH$



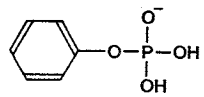
3-Phenylpyruvic acid (**14**) $R_1=R_2=R_3=H$



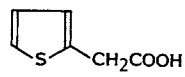
4-Phenylbutyric acid (**15**)



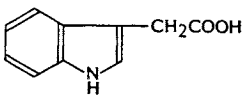
2-Phenylbutyric acid (**16**)



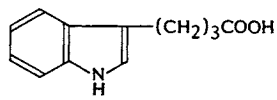
Phenylphosphate (**17**)



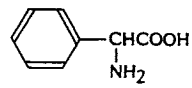
Thiophene-2-acetic acid (**18**)



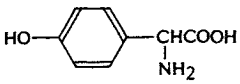
Indole-3-acetic acid (**19**)



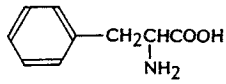
Indole-3-butyric acid (**20**)



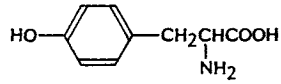
Phenylglycine (**21**)



4-Hydroxyphenylglycine (**22**)



Phenylalanine (**23**)



Tyrosine (**24**)

PA transport system although at different rates (Table 1). However, 3-hydroxyphenylacetic acid (**7**) and 4-hydroxyphenylacetic acid (**8**) did not cause induction, suggesting an important role for the position of the hydroxy group on the aromatic ring. Moreover, the fact that neither benzoic acid or its hydroxyl derivatives (**1**~**4**) nor phenylpropionic acid and its hydroxyl derivatives (**9**~**13**) induced the uptake of PA indicates that an even number of carbon atoms in the acyl chain bound to the aromatic ring is needed for induction. This could explain why the introduction of an oxygen atom between the aromatic ring and the acyl moiety (POA, **9**) leads to inactivity as an inducer. The notion that an even number of carbon atoms is needed is also supported by the fact that 4-phenylbutyric acid (**15**) is able to induce the PA transport system. However, with this molecule induction time was greatly delayed, suggesting that the true inducer is generated after phenylbutyric acid catabolism. The fact that after induction with phenylbutyric acid only benzylpenicillin is produced (isolated from the broths and evaluated by HPLC against standards of different penicillins[4,5]) (Table 1), indicates that phenylbutyric acid is converted by *P. chrysogenum* into phenylbutyryl-CoA and later by β-oxidation into the authentic inducer, phenylacetyl-CoA. The latter compound is a natural substrate of acyltransferase[1,7] which condenses it (by *N*-acylation) with 6-APA to form benzylpenicillin (penicillin G). Furthermore, analysis by GC of the side chain of the penicillin produced when phenylbutyric acid (0.1% w/v) was added to the fermentation broths showed that it was PA not phenylbutyric acid (see Fig. 3) suggesting that phenylbutyric acid must be transformed in phenylacetyl-CoA. These results agree well with those reported by us previously[8], showing that phenylbutyryl-CoA is not used as substrate by acyl-CoA: 6-APA acyltransferase whereas phenylacetyl-CoA is, however, very efficiently used. Furthermore, when 2-phenylbutyric acid (**16**) was tested, no induction was observed. Since this molecule cannot be catabolized by β-oxidation[9], phenylacetyl-CoA cannot be produced and the PA uptake system is not induced.

A further observation pointing to the need for a free moiety of acetyl-CoA in induction is that compounds containing an amino group in the α-position of the acyl group (D-α-aminophenylacetic acid and D-α-amino-4-hydroxyphenylacetic acid (**21** and **22** respectively) or closely related structural molecules such as phenylalanine and tyrosine (**23** and **24**) are not accepted as inducers. The presence of the NH_2 group in the side chain precursor molecule could cause steric hindrance that impedes access of these compounds to the active induction site.

When other molecules in which the phenyl ring had been substituted by other groups (thiophene-2-acetic acid, indole-3-acetic acid, indole-3-butyric acid) (**18**, **19** and **20**) were tested, no generation of the PA uptake system was observed, suggesting that the phenyl moiety also plays an important role in recognition of the

Fig. 3. GC obtained from: (A) The phenyl derivative released from the penicillin isolated from *Penicillium chrysogenum* Wis 54-1255 fermentation broths when 4-phenylbutyric (0.1% w/v) was supplied as side chain precursor; (B) 2-ethylhexanoic acid (2-EH).

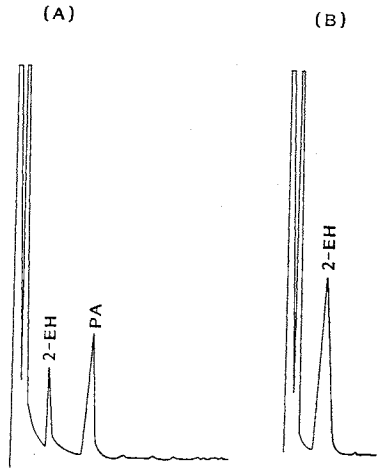


Table 1. Induction of PA transport system in *Penicillium chrysogenum* Wis 54-1255.

	Uptake rates (pmol/minute)			Type of β-lactam antibiotic produced after induction
	6 hours	12 hours	24 hours	
Control	11	14	20	6-APA; penicillins K and DF
+PA	800	2,200	3,200	Benzylpenicillin
+PA+glucose	12	16	460	6-APA; penicillins K, DF and benzylpenicillin
+PA+L-lysine	360	500	1,300	Benzylpenicillin
+2-Hydroxy-PA	395	904	1,964	2-Hydroxybenzylpenicillin (2-OHG)
+2-Hydroxy-PA+glucose	12	13	260	6-APA, penicillins K, DF and 2-OHG
+2-Hydroxy-PA+L-lysine	200	725	1,177	2-OHG
+4-Phenylbutyric acid (4-PB)	25	500	2,002	Benzylpenicillin
+4-PB+glucose	12	11	212	6-APA, penicillins K, DF and benzylpenicillin
+4-PB+L-lysine	14	389	1,680	Benzylpenicillin

Uptake rates were measured at 6, 12 and 24 hours after induction. In the three cases studied the level of glucose and L-lysine after 6, 12 and 24 hours of induction were 1.2, 0.02, 0% and 10, 2 and 0.5 mM respectively.

inducer by *P. chrysogenum*.

The PA transport system, induced by PA, 2-hydroxyphenylacetic acid and 4-phenylbutyric acid, is under catabolic regulation (Table 1) its appearance after induction being greatly delayed when glucose (2% w/v) was added to the broth 1 hour before the three inducer molecules. PA uptake was generated only when glucose was exhausted from the broths (Table 1)[10]. L-Lysine (50 mM), another molecule involved in the regulation of penicillin biosynthesis[11,12] also represses the synthesis of this transport system suggesting that the three molecules, PA, 2-hydroxyphenylacetic and 4-phenylbutyric acids, induce the same uptake system.

These data indicates that the PA uptake system is a regulatory point in the control of benzylpenicillin biosynthesis.

Acknowledgments

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