Metabolism of Tryptophan by *Pseudomonas aureofaciens* and Its Relationship to Pyrrolnitrin Biosynthesis

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(Received 29 February 1980; revised 21 May 1980)

Studies on the metabolism of tryptophan in *Pseudomonas aureofaciens* ATCC 15926 revealed different metabolic routes for the L- and D-isomer besides the biosynthetic pathway for pyrrolnitrin synthesis. L-Tryptophan catabolism follows the aromatic route via anthranilic acid. Tryptophan 2,3-dioxygenase was induced by L-tryptophan. Kynureninase and anthranilate 1,2-dioxygenase were induced by L-tryptophan, L-kynurenine and anthranilic acid. Anthranilate 1,2-dioxygenase was absent from a mutant strain of *P. aureofaciens* ATCC 15926 which produced about 30-fold increased amounts of pyrrolnitrin. The K_m values of tryptophan 2,3-dioxygenase and kynureninase did not differ substantially between the two strains. Kynurenine 3-monooxygenase, 3-hydroxyanthranilate 3,4-dioxygenase, tryptophanase and indolyl-3-alkane α -hydroxylase activities were not detected. D- and L-tryptophan were converted to indole-3-pyruvate by tryptophan aminotransferase and via indole-3-acetaldehyde to indole-3-acetic acid. This additional catabolic pathway as well as tryptophan racemase activity was constitutive and present in both strains.

INTRODUCTION

Tryptophan is the precursor of the antifungal agent pyrrolnitrin (Floss *et al.*, 1971; see Fig. 1) which is produced by several pseudomonads (Arima *et al.*, 1964; Elander *et al.*, 1968). Pyrrolnitrin, aminopyrrolnitrin and 3-chloroindole were isolated from cultures of *Pseudomonas aureofaciens* ATCC 15926 (Lively *et al.*, 1966).

During our studies on pyrrolnitrin biosynthesis, 4-(2-amino-3-chlorophenyl)pyrrole-2-carboxylic acid, 7-chloroindole-3-acetic acid, 3-chloroanthranilic acid, indole-3-acetic acid and anthranilic acid were identified in cultures of *P. aureofaciens* (Salcher *et al.*, 1980). The formation of these metabolites depended on the growth conditions (e.g. supplementation with D- or L-tryptophan). 4-(2-Amino-3-chlorophenyl)pyrrole-2-carboxylic acid was isolated from cultures grown in glycerol minimal medium containing 3 mM-D-tryptophan, whereas the enzyme activity for the formation of indole-3-acetamide or 7-chloroindole-3-acetamide was only present in cultures grown in glycerol minimal medium without any supplements. These results led us to question to what extent the pathways of tryptophan catabolism and pyrrolnitrin biosynthesis might compete for the same precursor, and how the D- or L-isomer of tryptophan would be metabolized. Furthermore, it was of interest to compare the enzyme pattern of tryptophan metabolism of the parent strain *P. aureofaciens* ATCC 15926 with the mutant strain ACN, which was obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Salcher & Lingens, 1980). This mutant strain produced about 30-fold increased amounts of pyrrolnitrin in a defined medium. Whereas the wild-type used L-tryptophan and anthranilic acid as sole sources of carbon, the mutant showed delayed

Fig. 1. Tryptophan metabolism in *Pseudomonas aureofaciens* wild-type ATCC 15926 and mutant ACN. The reaction catalysed by anthranilate 1,2-dioxygenase, which is absent from the mutant strain, is indicated by an asterisk. Trivial names of compounds: L-tryptophan (1), D-tryptophan (2), 7-chloro-L-tryptophan (3), 7-chloro-D-tryptophan (4), indole-3-acetamide (5), indole-3-pyruvic acid (6), indole-3-acetic acid (7), L-kynurenine (8), anthranilic acid (9), catechol (10), acetate (11), succinate (12), 4-(2-amino-3-chlorophenyl)pyrrole-2-carboxylic acid (13), 3-chloro-4-(2-amino-3-chlorophenyl)pyrrole-2-carboxylic acid (14), aminopyrrolnitrin (15), pyrrolnitrin (16).

growth with L-tryptophan and none with anthranilic acid (Salcher & Lingens, 1980). Our studies were therefore aimed also at the question of how increased production of secondary metabolites may be explained.

METHODS

Strains and growth conditions. *Pseudomonas aureofaciens* wild-type ATCC 15926 and mutant ACN were grown in a defined medium as described by Blumenstock *et al.* (1980). Carbon sources other than glycerol were sterilized by filtration and added at a final concentration of 3 mM.

Bacteria were grown at 30 °C with rotary shaking (100 rev. min⁻¹) in 2 l Erlenmeyer flasks containing 500 ml medium. The cultures were inoculated with 5 ml of a cell suspension taken from the exponential growth phase (approx. 1×10^8 cells ml⁻¹) and resuspended in 0.05 M-K/Na-phosphate buffer, pH 7.0. The bacteria were harvested in the late-exponential growth phase at a density corresponding to 2.5×10^8 viable cells ml⁻¹ or an $A_{400-600}$ of 1.5 in the Eppendorf photometer. The cells were washed twice with 0.05 M-K/Na-phosphate buffer, pH 7.0, and stored at -18 °C.

Pseudomonas fluorescens ATCC 29574, the reference strain for the indolyl-3-alkane α -hydroxylase test, was grown by the method of Roberts & Rosenfeld (1977) as well as under the conditions described above.

Preparation of cell extracts. Cells were disrupted by grinding with aluminium oxide (Blumenstock *et al.*, 1980) or by ultrasonication (Branson-Sonifier; 50 W, 6 $\times$ 30 s). For the latter method 1 g of frozen cells was suspended in 3 ml 0.05 M-K/Na-phosphate buffer, pH 7.0. After disruption and subsequent centrifugation at 30000 g (4 °C), the supernatant was dialysed against 100 vol. 0.05 M-K/Na-phosphate buffer, pH 7.0, for 5 h.

Protein determination. Protein concentration was determined by the methods of Layne (1957) and Lowry.

Enzyme assays. Anthranilate 1,2-dioxygenase (EC 1.14.12.1) was measured by the decrease in absorption at 310 nm due to the disappearance of the substrate anthranilate (Kobayashi & Hayaishi, 1970). The enzyme activity was lost after freezing the extracts.

3-Hydroxyanthranilate 3,4-dioxygenase (EC 1.13.11.6) was assayed by measuring the increase in absorption at 360 nm due to the production of 2-amino-3-carboxymuconate semialdehyde (Nishizuka *et al.*, 1970).

Activities of indole-3-acetaldehyde dehydrogenase and indole-3-pyruvate decarboxylase were determined according to the method of Fujioka *et al.* (1970).

Kynurenine 3-monooxygenase (EC 1.14.13.9) was assayed spectrophotometrically by measuring the decrease in absorption at 340 nm due to the oxidation of NADPH (Okamoto, 1970).

Kynureninase (EC 3.7.1.3) activity was measured spectrophotometrically by the decrease in absorbance at 365 nm (Knox, 1955).

Tryptophan 2,3-dioxygenase (EC 1.13.11.11) was assayed according to Ishimura (1970). The formyl-kynurenine formed was converted to kynurenine by the formamidase present in crude extracts and determined by the zinc acetate method (Yamamoto & Hayaishi, 1970). The interfering kynureninase activity in crude extracts was eliminated by preincubation with 5 mM-semicarbazide (Palleroni & Stanier, 1964).

Tryptophanase (EC 4.1.99.1) activity was measured by colorimetric determination of the indole formed (Morino & Snell, 1970).

Indolyl-3-alkane α -hydroxylase was assayed by the method of Narumiya *et al.* (1979).

The purity of the D- and L-tryptophan used was tested polarographically with L-amino acid oxidase (EC 1.4.3.2) and D-amino acid oxidase (EC 1.4.3.3) according to the method of Wellner (1971) or Yagi (1971).

Tryptophan racemase was tested according to Behrman (1962) and tryptophan aminotransferase (EC 2.6.1.27) by the method of Fujioka *et al.* (1970).

Thin-layer chromatography. The production of metabolites during growth and during the enzyme reactions was also followed by thin-layer chromatography (Salcher & Lingens, 1980).

Determination of extracellular L-tryptophan. The production of extracellular L-tryptophan was analysed (Snell, 1957) in minimal medium containing 3 mM-D-tryptophan. Samples (10 ml) were taken from the cultures during growth and centrifuged at 5000 g. The supernatants were sterilized by filtration and assayed microbiologically using *Lactobacillus arabinosus* ATCC 8014 as the test organism.

Reagents. These were of the highest purity grade available. L-Tryptophan and anthranilic acid were obtained from Merck. D-Tryptophan, 3-hydroxyanthranilic acid, L-3-hydroxykynurenine, L-kynurenine sulphate, indole-3-acetic acid and the remaining indole compounds were obtained from Sigma. L-Amino acid oxidase (snake venom) and D-amino acid oxidase (hog kidney) were purchased from Boehringer.

RESULTS AND DISCUSSION

The aromatic pathway of tryptophan catabolism

Kynurenine is a branchpoint metabolite of tryptophan catabolism in pseudomonads since two alternative routes diverge from it: the aromatic pathway via anthranilic acid and the quinoline pathway via kynurenic acid (Behrman, 1962). *Pseudomonas aureofaciens* ATCC 15926 was unable to use D-tryptophan and kynurenic acid as sole carbon sources, whereas anthranilic acid was metabolized (Salcher & Lingens, 1980). Tryptophan catabolism through L-kynurenine in *P. aureofaciens* follows the aromatic pathway via anthranilic acid (Fig. 1). Tryptophan 2,3-dioxygenase, the first enzyme of this pathway, is induced by L-tryptophan but not by L-kynurenine (Table 1). This is in contrast to the situation in *P. fluorescens* where the latter compound induces tryptophan 2,3-dioxygenase (Palleroni & Stanier, 1964).

In the following studies the effect of D-tryptophan and of glycerol on the induction of catabolic enzyme synthesis was examined. The cultures were grown in the presence of 3 mM-D-tryptophan together with 3 mM-glycerol, because *P. aureofaciens* was unable to use D-tryptophan as sole carbon source. The same culture conditions were applied in other growth studies with L-tryptophan, L-kynurenine and anthranilic acid, each at 3 mM. Catabolic enzyme activities of bacteria grown in the presence of 3 mM-D-tryptophan were within the range characteristic of uninduced bacteria (Table 1). Tryptophan 2,3-dioxygenase, kynureninase and anthranilate 1,2-dioxygenase were induced by L-tryptophan in the presence

Table 1. *Induction of enzymes of the aromatic pathway of tryptophan catabolism in Pseudomonas aureofaciens wild-type ATCC 15926 and mutant ACN*

Enzyme activities were determined as described in Methods and are expressed as $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

Strain	Carbon source added to minimal medium (3mm)	Enzyme activity		
		Tryptophan 2,3-dioxygenase	Kynureninase	Anthranilate 1,2-dioxygenase
Wild-type ATCC 15926	Anthranilic acid	0.02	4.3	3.2
	Glycerol	0.09	0.9	0.83
	L-Kynurenine	0.08	8.2	6.8
	D-Tryptophan + glycerol	0.08	1.2	0.95
	L-Tryptophan	3.1	8.5	7.2
Mutant ACN	Anthranilic acid	0.03	2.2	0
	Glycerol	0.03	0.7	0
	L-Kynurenine	0.07	5.1	0
	D-Tryptophan + glycerol	0.09	0.5	0
	L-Tryptophan	2.9	4.7	0

Table 2. *Comparison of the K_m values of anthranilate 1,2-dioxygenase, kynureninase and tryptophan 2,3-dioxygenase from Pseudomonas aureofaciens wild-type ATCC 15926 and mutant ACN*

Enzyme activities were assayed as described in Methods using different substrate concentrations. K_m values were determined from Lineweaver-Burk plots.

Enzyme	K_m value (μM)	
	Wild-type ATCC 15926	Mutant ACN
Anthranilate 1,2-dioxygenase	120	No activity
Kynureninase	250	130
Tryptophan 2,3-dioxygenase	650	460

of glycerol (results not shown) to the same extent as in its absence (Table 1), indicating that these enzymes were not repressed by glycerol. In contrast to the results obtained with tryptophan 2,3-dioxygenase, the enzymes kynureninase and anthranilate 1,2-dioxygenase were induced by L-kynurenine and anthranilic acid as well as L-tryptophan (Table 1). These results suggest a sequential induction pattern for tryptophan catabolic enzymes in *P. aureofaciens* ATCC 15926 and the mutant strain. Anthranilate 1,2-dioxygenase activity could not be detected in extracts of the mutant strain. Activities (Table 1) and K_m values (Table 2) of tryptophan 2,3-dioxygenase and kynureninase were similar in the two strains.

When L-kynurenine was replaced by L-3-hydroxykynurenine in the kynureninase assay, enzyme activities decreased to 10 %. The wild-type strain had an enzyme activity of $0.8 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ and the mutant $0.6 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ when the bacteria were grown in minimal medium containing L-tryptophan as sole carbon source. Similar results for the substrate specificity of kynureninase were obtained with *P. fluorescens* by Gaertner & Shetty (1977). In *P. aureofaciens* no kynurenine 3-monooxygenase or 3-hydroxyanthranilate 3,4-dioxygenase activity was detected. The route for synthesizing NAD from L-tryptophan is thus not complete. Representatives of the related genus *Xanthomonas* are known to synthesize NAD from L-tryptophan (Lingens & Vollprecht, 1964; Lingens *et al.*, 1966) and contain an inducible kynureninase (Shetty & Gaertner, 1978).

A novel tryptophan-metabolizing enzyme, indolyl-3-alkane α -hydroxylase, was isolated from *P. fluorescens* ATCC 29574 (Roberts & Rosenfeld, 1977), but neither this enzyme nor tryptophanase could be detected in *P. aureofaciens*.

Table 3. Influence of L-tryptophan and D-tryptophan on the tryptophan aminotransferase activity in *Pseudomonas aureofaciens* wild-type ATCC 15926 and mutant ACN

Bacteria were grown in glycerol minimal medium with or without 3 mM-L- or D-tryptophan. Tryptophan aminotransferase activity was determined as described in Methods. Activities are expressed as $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$.

Supplement to glycerol minimal medium (3 mM)	Substrate used in aminotransferase assay	Enzyme activity	
		Wild-type ATCC 15926	Mutant ACN
D-Tryptophan	L-Tryptophan	0.59	0.46
L-Tryptophan	L-Tryptophan	1.2	0.84
None	L-Tryptophan	0.35	0.49
D-Tryptophan	D-Tryptophan	0.23	0.18
L-Tryptophan	D-Tryptophan	0.22	0.21
None	D-Tryptophan	0.14	0.19

Table 4. Activities of enzymes of the indole-3-acetic acid pathway in *Pseudomonas aureofaciens* wild-type ATCC 15926 and mutant ACN

Enzyme activities were assayed as described in Methods and are expressed as nmol min^{-1} (mg protein) $^{-1}$.

Strain	Carbon source added to minimal medium (3 mM)	Enzyme activity	
		Indole-3-pyruvate decarboxylase	Indole-3-acetaldehyde dehydrogenase
Wild-type ATCC 15926	Glycerol	5.1	8.3
	L-Tryptophan	6.8	10.4
	D-Tryptophan + glycerol	7.2	10.0
Mutant ACN	Glycerol	6.5	9.2
	L-Tryptophan	4.6	7.8
	D-Tryptophan + glycerol	4.3	10.9

The indole-3-acetic acid pathway

In *P. aureofaciens* a further pathway of tryptophan metabolism is available in synthesizing the phytohormone indole-3-acetic acid, which is also produced by the phytopathogenic species *P. syringae* (*P. savastanoi*) (Kuo & Kosuge, 1969). In plants there are several pathways leading from tryptophan to indole-3-acetic acid: the tryptamine pathway, the indole-3-acetaldoxime pathway, the tryptophol pathway and the indole-3-pyruvate pathway (Schneider & Wightman, 1974). The last pathway is probably present in *P. aureofaciens* (see Fig. 1). Indole-3-pyruvic acid was detected in the tryptophan aminotransferase assay. The enzyme activity was enhanced in extracts of cells grown in the presence of 3 mM L-tryptophan and when they were assayed with L-tryptophan as substrate (Table 3), whereas the aminotransferase activity with D-tryptophan as substrate was within the range of the activity obtained with L-tryptophan as substrate from cells grown in glycerol minimal medium. In both strains indole-3-pyruvate was metabolized via indole-3-acetaldehyde to indole-3-acetic acid. Indole-3-pyruvate decarboxylase and indole-3-acetaldehyde dehydrogenase (Table 4) as well as tryptophan aminotransferase were constitutive. In neither strain were the activities of L- or D-tryptophan racemase influenced by supplements to the growth medium; the enzyme activities were about $0.3 \mu\text{mol min}^{-1}$ (mg protein) $^{-1}$ throughout. In order to examine the extent to which the conversion of D-tryptophan to the L-isomer contributed to the extracellular concentration of L-tryptophan, cultures were grown in glycerol minimal medium supplemented with 3 mM-D-tryptophan ($612 \mu\text{g ml}^{-1}$) and the extracellular concentration of L-tryptophan was determined. The maximum concentration, reached in the stationary growth phase, was $0.7 \mu\text{g L-tryptophan ml}^{-1}$ for both strains.

Similar concentrations were detected in cultures grown in glycerol minimal medium without supplements.

Tryptophan catabolism and pyrrolnitrin biosynthesis

Pyrrolnitrin is synthesized by *P. chlororaphis*, *P. cepacia* (*P. multivorans*) and several *P. aureofaciens* strains. These organisms produced different amounts of pyrrolnitrin when the growth medium (complete medium) was supplemented with D- or L-tryptophan (Elander *et al.*, 1968). The strain-dependent response, which is not a question of the uptake of tryptophan, might be due to different pathways of tryptophan catabolism which compete with the pyrrolnitrin biosynthetic route for the same precursor. Although D-tryptophan enhanced and L-tryptophan depressed pyrrolnitrin production in *P. aureofaciens*, the role of L-tryptophan as a possible precursor should not be excluded, because it may be masked by extensive L-tryptophan catabolism. The mutant strain, which lacks anthranilate 1,2-dioxygenase activity, is more efficient in pyrrolnitrin production.

In cultures of the wild-type and the mutant strain small amounts of 7-chloroindole-3-acetic acid and 3-chloroanthranilic acid were detected (Salcher & Lingens, 1978). The presence of these metabolites suggests the existence of 7-chlorotryptophan. Also 4-(2-amino-3-chlorophenyl)pyrrole-2-carboxylic acid, a possible intermediate of pyrrolnitrin biosynthesis, might be derived from 7-chlorotryptophan (Salcher *et al.*, 1978). In addition, the tryptophan 2,3-dioxygenase activity was fivefold lower with 7-chloro-L-tryptophan [$0.6 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$] instead of L-tryptophan as a substrate (Table 1) when both strains were grown with L-tryptophan as the sole carbon source. These results suggest that pyrrolnitrin biosynthesis and tryptophan catabolism diverge in an early step when 7-chlorotryptophan is produced from tryptophan.

We thank Susanne Weiss for skilful technical assistance, Karl-Heinz van Pée for a sample of 7-chloro-L-tryptophan and the Fonds der Chemischen Industrie for support.

This paper is dedicated to Professor K. Mothes on the occasion of his 80th birthday.

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