CHARACTERIZATION OF TRYPTOPHAN AUXOTROPHS FROM *PSEUDOMONAS AERUGINOSA*

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Abstract: Four tryptophan auxotrophs were isolated from *Pseudomonas aeruginosa*. From the physiological and antibiotic tests, we can conclude these four mutants are independent. However, they can be grouped into two categories according to the cross-feeding data and growth tests: Two were partially blocked on tryptophan synthetase, and the other two were inhibited before indole-glycerol phosphate formation.

INTRODUCTION

The five clustered genes of the tryptophan biosynthetic pathway in *Escherichia coli* (genes E, D, C, B, and A) have been shown to be arranged in the same order of occurrence as the biosynthetic reactions which they control (Yanofsky *et al.*, 1959). The synthesis of these biosynthetic enzymes is normally repressed in the presence of the end product (Gibson *et al.*, 1969). However, the genes for tryptophan biosynthesis in *Pseudomonas aeruginosa* & *P. putida* do not form a single cluster but were located in three separate transduction linkage groups (Fargie *et al.*, 1965). Also, the regulation of this pathway follows a different pattern. Crawford and Gunsalus (1966) found in *Pseudomonas putida* that *trp* ABD enzymes for steps 1, 2 and 4 of the tryptophan biosynthetic pathway were repressed by tryptophan, while the enzymes of *trp* C and *trp* EF linkage groups were unaffected.

Several analogues of tryptophan and its biosynthetic intermediates have been used to test the effect on the growth and on the tryptophan synthetase activity of *P. aeruginosa* in this laboratory (Tsai *et al.*, 1975; Ho *et al.*, 1976). In order to study the regulatory mechanism thoroughly, auxotrophs can be used as a very important tools. In this report we isolate some tryptophan auxotrophs for the studies.

MATERIALS AND METHODS

Bacteria

Pseudomonas aeruginosa strain 1 (designated as PA-1) was used as the wild type in these studies. Mutants were isolated as described below and were numbered as 1-5, 1-11, 3-11, and 5-15.

Cultivation of bacteria

Vogel and Bonner's (1956) minimal medium supplemented with 0.2% glucose was used for the studies. One point five per cent agar was addded for the preparation of plates. All the carbon sources were sterilized separately and added to the desired concentration.

Isolation of mutants

Wild type cells were treated with 100 μg/ml of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in glucose minimal medium supplemented with tryptophan for 15 minutes (Aldeberg *et al.*, 1965), then enriched with 1.5 mg/ml d-cycloserine (DCS) for 4 hours in glucose minimal medium. Tryptophan auxotrophs were isolated by replica plating methods (Lederberg, 1952).

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Cross-feeding

Cells were grown on nutrient agar slants and a small inoculum of one mutant was streaked vertically on plates of glucose minimal agar near the edge of each plate; then the other mutants to be tested were streaked horizontally on the plate just up to the line of the first mutant, care being taken not to touch the inoculum of the other mutants. All mutants were tested in all possible combinations following the pattern shown below:

Growth curve

The mutant and wild type cells were washed with sterilized saline from overnight growth on an agar slant into glucose minimal medium with the addition of 20 μg/ml tryptophan or indole and incubated at 37°C in the reciprocal shaker with 128 vibration per minute. Cell turbidity was measured by Bauch and Lomb Spectronic 20 at 540 nm.

Crude extract preparation and enzyme assay

Strains to be assayed for tryptophan synthetase were prepared as previously described (Ho and Liu, 1976). The enzyme activity was assayed according to the method of Smith and Yanofsky (1962) in phosphate buffer. One unit of enzyme activity is defined as the consumption of 0.1 μ mole indole in 20 minutes at 37°C. Protein concentration was determined by the method of Lowry *et al.* (1951).

Detection of indole accumulation

The wild type and mutant cells were grown in glucose minimal medium supplemented with tryptophan. During different time intervals, 5 ml of cells were collected and centrifuged to remove debris. The amount of indole excreted into the medium by the bacteria was determined according to the method of Smith & Yanofsky (1962).

RESULTS

The spontaneous mutation rate is very low, especially for a special character, therefore the treatment with a mutagen is necessary. Four tryptophan auxotrophs were isolated by using the method described before from the tryptophan containing glucose minimal medium and numbered as 1-5, 1-11, 3-11 and 5-15.

Cross-feeding

According to the theory of cross-feeding each mutant may build up high levels of intermediate before its own block and may excrete this intermediate into the medium. The intermediate diffuses slightly through the agar and is then available to the other mutants for growth. Only those mutants which are blocked in a step prior to the intermediate are able to use it and grow. Any mutant that can be fed by another mutant will have a block in its pathway before that of the mutant which is able to feed it. Cross-feeding data gives an indication of the order of the steps in a pathway. According to Table 1, strain 1-5 and 3-11 were fed by 1-11 and 5-15 indicated that the latter two mutants were blocked at a point in the pathway subsequent to that at which the former mutants were blocked.

Growth studies

Four mutants were tested to grow on glucose minimal medium supplemented with anthranilic acid, indole or tryptophan (Table 2). They did not grow on anthranilic acid-supplemented glucose minimal medium, but did grow on tryptophan-supplemented glucose minimal medium. From the data shown in Table 2, we could conclude that strain 1-11 and 5-15 were partially

Table 1. Cross-feeding experiments

Fed by	Cells in agar			
	1-5	3-11	1-11	5-15
1-5		−	+	+
3-11	−		+	+
1-11	+	+		
5-15	+	+	−	−

+: cell growth; −: no growth.

Table 2. Growth of PA-1 and tryptophan auxotrophs on different growth medium

Glucose minimal medium supplemented with 20 μg/ml of	Days	Strains				
		wild type	1-5	1-11	3-11	5-15
	1st and 2nd day	##	−	−	−	−
anthranilic acid	1st day	##	−	−	−	−
	2nd day	##	−	−	−	−
indole	1st day	##	+	−	##	−
	2nd day	##	+	+	##	+
tryptophan	1st day	##	##	##	##	+
	2nd day	##	##	##	##	+

+: Growth; −: No growth.

blocked at tryptophan synthetase, because they could use indole on the second day but not on the first day. Strain 1-5 and 3-11 were apparently blocked at the steps before indoleglycerol formation. The growth curve of the wild type and mutants on tryptophan-glucose and indole-glucose minimal medium are shown in Fig. 1 and Fig. 2. Strain 1-11 and 5-15 growing on indole-glucose minimal medium show a very long lag period before they start to grow.

Physiological characteristics

We conducted the following experiments: indole production, nitrate reduction, citrate and carbohydrate utilization, starch and gelatin hydrolysis. litmus milk reaction, oxidase and catalase production test to compare the mutants with the wild type cells. We found the two mutant strains 1-5 and 3-11 differed from the parental strain and the other two mutant strains 1-11 and 5-15 only in the production of indole and H_2S (Table 3). All the mutants could use citrate but strains 5-15 and 3-11 used it very slowly.

Antibiotics sensitivities of mutants

Four auxotrophs and wild type cells were tested on several kinds of antibiotics. They were sensitive to some antibiotic such as streptomycin, aureomycin, dedomycin, tetramycin; and were resistant to leucomycin, erythromycin, ampicillin, sulfamerazine, kanamycin, oleandomycin. However, they also reacted differently with other antibiotics (Table 4).

Indole accumulation

In the experiment of indole accumulation, wild type and two auxotrophs 1-5 and 3-11

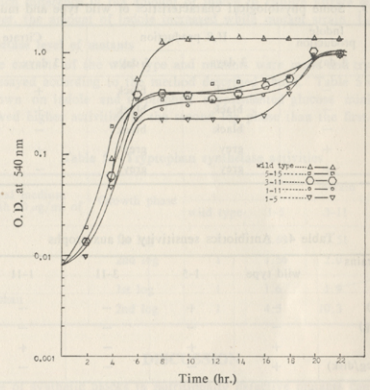

Fig. 1. Cell growth on tryptophan glucose minimal medium.

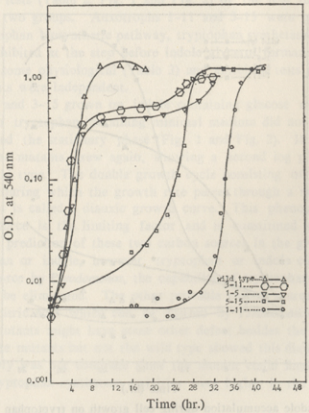

Fig. 2. Cell growth on indole glucose minimal medium.

Table 3. Some physiological characteristics of wild type and mutants

experiment / strain	Indole production	H_2S production		Citrate utilization	
	3 days	3 days	5 days	3 days	5 days
PA-1	−	black	black	+	+
1-5	−	black	black	+	+
3-11	−	black	black	−	+
1-11	+	grey	grey	+	+
5-15	+	grey	grey	−	+

Table 4. Antibiotics sensitivity of auxotrophs

strains / antibiotics	wild type	1-5	3-11	1-11	5-15
chloromycin (5 mcg)	−	+	−	−	+
sulfathiazole (50 mcg)	−	−	−	−	+
colistin (100 μ/disk)	+	+	−	+	+
cephaloridine (30 mcg/disk)	+	−	−	−	+

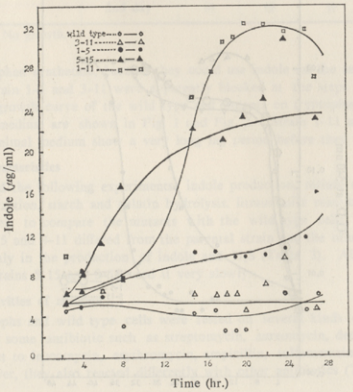

Fig. 3. Indole accumulation during cell growth on tryptophan glucose minimal medium.

did not show much difference in the amount of indole in culture filtrate during growth cycle (Fig. 3). However, the amount of indole increased while mutant strain 1-11 and 5-15 grew.

Tryptophan synthetase level of mutants

The cell free extracts of the wild type and mutants were made and tryptophan synthetase activities were assayed according to the method described above. Table 5 showed the enzyme level of cells grown on indole and tryptophan supplemented glucose minimal medium. All the mutants showed higher activities in the second log phase than the first.

Table 5. Tryptophan synthetase activities

Glucose minimal medium supplemented with 20 μg/ml of	Growth phase	Strain				
		wild type	1-5	3-11	1-11	5-15
indole	1st log	1	0.69	0.51		
	2nd log	1	1.56	2.0	1.9	1.6
tryptophan	1st log	1	1.6	1.9	2.4	2.0
	2nd log	1	4.3	10.3	7.7	3.7

DISCUSSION

The sequence of synthetic blocks in nutritionally defective mutants can be determined by cross-feeding tests (Hayes, 1968). From the cross-feeding data (Table 1) and growth tests (Table 1) and growth tests (Table 2, Fig. 1 and Fig. 2) of the four mutants we isolated, we can divide them into two groups. Auxotrophs 1-11 and 5-15 were partially blocked at the last step of the tryptophan biosynthetic pathway, tryptophan synthetase. However, auxotrophs 3-11 and 1-5 were inhibited at the step before indole glycerol formation. Since four mutants reacted differently to some physiological (Table 3) and antibiotic tests (Table 4), we concluded that these four mutants were independent.

The mutants 1-5 and 3-15 grown on indole containing glucose minimal medium and all the mutants grown on tryptophan-containing minimal medium did not show complete growth when they first reached the stationary phase (Fig. 1 and Fig. 2). If the flasks were shaken for a longer time, the mutants grew again, showing a second log phase and reached nearly the plateau of the wild type. The double growth cycle consisting of two exponential phases separated by a phase during which the growth rate passes through a minimum, even becoming negative in some cases is called a diauxic growth curve. This phenomemon occurs in media where the organic source is the limiting factor and is constituted of two carbon sources (Dawson, 1974). The prediction of these two carbon sources in the growth medium could be glucose and tryptophan or indole, however, tryptophan or indole can not be used as sole carbon and energy source by *Pseudomonas*, the capability of tryptophan or indole as the second carbon source should be eliminated. The other probable carbon source in Vogel and Bonner's minimal medium is citric acid which can be utilized by *Pseudomonas* as a sole carbon and energy source. The mutants might have some other defect besides the tryptophan biosynthetic pathway since only the mutants but not the wild type showed this diauxic growth curve. This kind of defect probably was not complete since the mutant could finally grow as well as the wild type, and the tryptophan synthetase activities were higher in the second log phase than the first (Table 5).

The odor of indole appeared strongly in the flasks of strain 1-11 and 5-15 during the

growth on tryptophan containing minimal medium, therefore the amount of indole in the culture filtrate was determined. Fig. 3 showed the concentration of indole of strain 1-11 and 5-15 had increased while they grew. However, when the second log phase started, the concentration of indole had a tendency to decrease. Since the strains 1-11 and 5-15 were concluded to be partially blocked at the step of tryptophan synthetase, the synthesis of tryptophan biosynthetic enzymes, except for tryptophan synthetase, could proceed during the growth period and the tryptophan biosynthetic intermediates could accumulate especially indole-glycerol phosphate. If the mutation occurring at the tryptophan synthetase step was due to the slow synthesis of tryptophan synthetase, or the low activity of this enzyme, the accumulated indole-glycerol phosphate could still be converted to indole and triose phosphate by the tryptophan synthetase A subunit and the excess amount of indole would excrete into the culture filtrate. Gunsalus (1967) found in *Pseudomonas* tryptophan synthetase was induced by its substrate, indoleglycerophosphate. Since the tryptophan synthetase was only partially blocked and could be induced slowly by indoleglycerophosphate. The amount of indole in strains 1-11 and 5-15 culture filtrate decreased after the second log phase (Fig. 3) and enzyme activities increased during the second log phase (Table 5).

Ho *et al.* (1976) showed that both 5-fluoro-indole and 5-fluoro-tryptophan could inhibit the growth of PA-1. However 5-fluoro-indole seemed not to inhibit PA-1 by it conversion to 5-fluoro-tryptophan. Further experiments could be done by using the tryptophan auxotrophs isolated in this report.

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