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Enzymology and regulation of the atropine metabolism in pseudomonas putida

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CHAPTER 4

BREAKDOWN OF ATROPINE AND TROPIC ACID

IN PSEUDOMONAS PMBL-1

4.1 INTRODUCTION

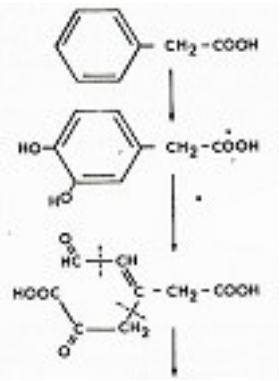
The genus Pseudomonas has the striking ability to metabolize many aromatic compounds and to use those as source of energy. For a survey, see review papers by van der Linden and Thijsse 1965 and Gibson 1968. Typical examples are benzoic acid and 6-chloroxylenol used on a large scale as preservative and disinfectant respectively.

The breakdown of aromatic compounds in Pseudomonas species may start by a hydroxylation of the aromatic ring, whereas in other cases the side chain is broken down firstly, either partially or completely.

The degradation of the aromatic ring by hydroxylation (fig 4.1) results in the formation of the di-phenol, followed by insertion of an oxygen molecule to produce an unsaturated more basic fatty acid. Finally, decomposition products

Fig 4.1

Possible metabolic pathway in Pseudomonas for breakdown of phenylacetic acid through 3,4 dihydroxyphenylacetic acid into puruvic,acid, acetoacetic acid, formic acid.



are formed consisting of 2-4 carbon atoms, which can be used by the bacterium as building blocks for the synthesis of essential components of the cell. The positioning of the hydroxyl groups and the oxygen molecule in the aromatic ring appears to be different for various Pseudomonas species; fig. 4.1 shows only one of the possibilities. This type of breakdown has been shown for the aromatic compounds benzoic acid, phenylacetic acid, 3-phenylpropionic acid and thymol (2-hydroxy-3-isopropyl toluene) (Chamberlain and Dagley 1968).

In cases of breakdown of the side chain first, this occurs usually by oxidation and decarboxylation. For example, in another Pseudomonas putida, mandelic acid (2-hydroxyphenylacetic acid) is converted into benzoic acid (Gunsalus et al. 1953 a and b; Stanier et al. 1953); n-butylbenzene is oxidized to phenylacetic acid.

Both patterns have been observed in the breakdown of p-cresol (p-hydroxy-toluene) (Chapman and Hopper 1968); some Pseudomonas species oxydize this compound to p-hydroxyphenylacetic acid (degradation of the side chain), whereas in other Pseudomonaceae p-cresol is converted by hydroxylation of the phenyl ring into 2.3 dihydroxytoluene.

There is no connection between the chemical structure of a compound and its metabolic pathway in a certain type of bacterium. Therefore, it was not possible to predict the most probable breakdown of tropic acid in the Pseudomonas strain isolated by Rörsch and Berends (MBL).

In the research described in this chapter, the adaptation of PMBL-1 to some aromatic carboxylic has been studied to determine whether these compounds are closely related to the metabolism of tropic acid. In addition, it has been tried to identify degradation products of tropic acid in the growth medium of wild type and mutants of PMBL-1. The results obtained suggest that in the metabolism of tropic acid the aliphatic side chain is broken down firstly.

4.2 CARBON SOURCES FOR PMBL-1

A number of compounds has been studied as possible carbon source in order to get some idea on the capacity of Pseudomonas PMBL-1 to metabolize aromatic and atropine-like compounds. Pseudomonas PMBL-1 was cultivated in a synthetic medium with 0.1% glucose or succinic acid and 0.025% of the compound to be investigated. This culture was used to inoculate fresh synthetic medium with 0.025% of the compound under study and 0.005% succinic acid. This culture was incubated at 30^0. After 18 hours, the concentration of the bacteria was assayed according to chapter 2.4.

The following compounds can be used as carbon source:

atropine	phenylacetic acid	phenylglyoxlic acid
homatropine	p-hydroxyphenylacetic acid	phenylacetaldehyde
tropine	m-hydroxyphenylacetic acid	benzaldehyde
tropic acid	phenylpyruvic acid	

These compounds can serve as carbon source in higher concentrations as well, except phenylacetaldehyde and benzaldehyde (toxic above 0.025%).

None or very limited growth was observed with the following compounds:

2-phenylacrylic acid	3-phenylacrylic acid	2-phenylpropanol
2-phenyl-2-hydroxypropionic acid	2-phenylpropionic acid	3-phenylpropanal
2-hydroxy-3-phenylpropionic acid	2-phenylpropanal	2-phenylethanol
2-hydroxyphenylacetic acid	benzyl alcohol	

An extended list of compounds that can or cannot be metabolized see table 10.3.

4.3 THE ADAPTATON OF PMBL-1 FOR AROMATIC ACIDS

The adaptation of Pseudomonas PMBL-1 has been studied for tropic acid and several other aromatic acids. One of the aspects investigated concerned the simultaneous adaptation to tropic acid and other compounds. In this way it could be possible to observe relationships between the metabolism of these aromatic acids without actual knowledge of their breakdown pathway in the bacterium.

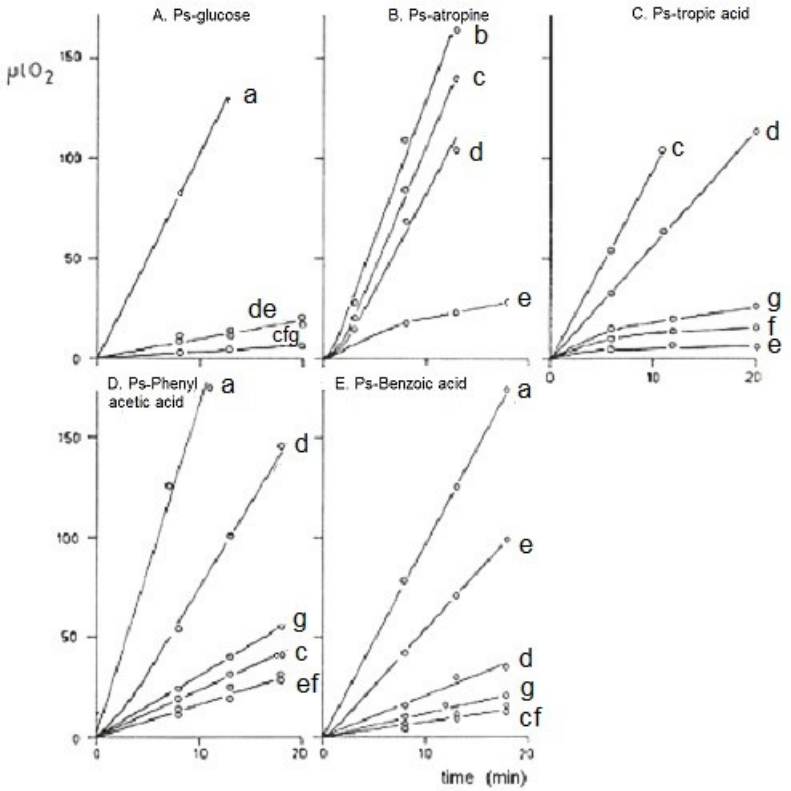
In such a study, one can use the feature of bacteria, which have been adapted to a certain carbon source, to be able to metabolize this compound immediately upon its addition to the medium. Non-adapted bacteria need an adaptation period before the metabolism can proceed. A precise distinction between metabolizing and non-metabolizing cells can be made for obligate aerobic organisms by measuring the uptake of oxygen as a function of time (Stanier 1950).

This method has been used to study adaptation of PMBL-1 (obligate aerobic see chapter 2.3) for the following carbon sources: glucose, atropine, tropic acid, phenylacetic acid, p-hydroxyphenylacetic acid and m-hydroxyphenylacetic acid, benzoic acid and glucose. The uptake of oxygen gas has been quantified using the manometric technique according to Warburg (chapter 2.6).

The results are presented in fig. 4.2.A - E. The oxygen consumption observed after addition of the carbon source specified (at time t=0) is plotted against time. Fig.4.2 shows that Pseudomonas cultivated with glucose as carbon source (Ps-glucose) can metabolize only glucose right away.

Fig 4.2 A-E

Oxygen uptake by PMBL-1



A. glucose
B. atropine
C. tropic acid
D. phenylacetic acid
E. benzoic acid

a. glucose
b. atropine
c. tropic acid
d. phenylacetic acid
e. benzoic acid
f. p-hydroxyphenylacetic acid
g. m-hydroxyphenylacetic acid

PMBL-1 grown on various carbon sources A-E. Ps-glucose is PMBL-1 grown with glucose as carbon source etc. On time t=0 various compounds a-g were added. Oxygen consumption was measured. Data were corrected for the spontaneous oxygen consumption (0.5-1.5 µl/min). Experimental details are documented in chapter 2.6.

Pseudomonas PMBL-1 cultivated with atropine as carbon source (Ps-atropine) starts to consume oxygen immediately after addition of atropine, tropic acid and phenylacetic acid, but not after addition of benzoic acid (fig. 4.2 B). According to expectation, Ps-atropine is adapted for tropic acid. Ps-atropine appears to be adapted to phenylacetic acid as well, suggesting either phenylacetic acid is metabolized by the same enzymes or phenylacetic acid is an intermediate in the breakdown of atropine.

The oxygen consumption by Ps-tropic acid (fig.4.2.C) confirms the experiment with atropine. These bacteria show adaptation not only to tropic acid but to phenyl acetic acid as well. These bacteria show no or only a little metabolic activity immediately after addition of benzoic acid, m-hydroxy- or p-hydroxyphenylacetic acid.

Fig. 4.2.D shows the oxygen uptake by Ps-phenylacetic acid: the bacteria have been adapted for phenylacetic acid and for glucose, but incubation with tropic acid, m-hydroxy- and p-hydroxyphenylacetic acid does not result in immediate oxygen consumption. The results shown in fig. 4.2.E suggest that after growth with benzoic acid as carbon source only this compound and glucose are metabolized straight away. The oxygen uptake after addition of glucose in this and the previous culture suggest that the enzymes involved in the breakdown of glucose are present in the cells irrespective the presence of glucose in the medium used for cultivation of the bacteria.

These adaptation studies show that the system for the breakdown of atropine and tropic acid is only present in Ps-atropine and Ps-tropic acid. Ps-atropine and Ps-tropic acid are fully adapted for phenylacetic acid. However, tropic acid is not immediately metabolized by Ps-phenylacetic acid. This suggests that the adaptation of Ps-tropic acid for phenylacetic acid is not due to the use of the same enzymes, but due to phenylacetic acid being an intermediate in the breakdown of atropine and tropic acid.

4.4 INVESTIGATION WITH MUTANTS, BLOCKED IN THE BREAKDOWN OF TROPIC ACID.

Parallel to the study of the adaptation of PMBL-1, a study of the metabolism of tropic acid was undertaken in PMBL-1 and in mutants by a search for the presence of degradation products in the cultivation medium. This was done after cultivation in synthetic medium with tropic acid as sole carbon source.

After cultivation, the medium was extracted and analyzed (chapter 2.7) by thin layer chromatography. An experiment with the wild type PMBL-1 failed. Thin layer chromatography only confirmed the consumption of tropic acid by the bacteria. In a next step, mutants were isolated from PMBL-1, not able to breakdown tropic acid anymore. Several dozens of mutants of PMBL were

obtained unable to grow with atropine or tropic acid as sole source of carbon. These mutants were still able to metabolize tropine, phenylacetic acid or glucose: phenotype Atr^{-} Tro^{-} Trp^{+} Pac^{+} Glu^{+}.

It seemed not unreasonable to assume that amongst these mutants some still are able to carry out a partial breakdown of tropic acid. Some might even be able to excrete an intermediate in the tropic acid breakdown when cultivated in the presence of tropic acid with glucose as carbon source.

The mutants were cultivated in a synthetic medium with 0.2% glucose and 0.2% tropic acid. The cultures were shaken at 30^0 during 3 days. Thereafter, the growth medium was analyzed using thin layer chromatography. However, none of the mutants showed accumulation of degradation products of tropic acid in the cultivation medium to such an extent that those products could be detected.

4.5 MUTANTS, BLOCKED IN THE METABOLISM OF PHENYLACETIC ACID AND p-HYDROXYPHENYLACETIC ACID

Since the investigation of Tro^{-} mutants did not result in new information on the breakdown of tropic acid, it was tried to isolate mutants unable to breakdown phenylacetic acid. The breakdown of this compound is related to that of tropic acid as concluded from the adaptation study (4.3). So, a study of the metabolism of Pseudomonas blocked in the metabolism of phenylacetic acid (Pac^{-}) might provide information about the breakdown of tropic acid.

PMBL-1 was treated with a mutagenic agent (chapter 2.5). The treated bacteria were selected for the phenotype Pac^{-}. Simultaneously, mutants were selected blocked in the metabolism of p-hydroxyphenylacetic acid (Php^{-}).
Amongst about 10.000 colonies isolated, a total of 52 mutants were found. Their phenotypes have been listed in table 4.3. The table shows that all Pac^{-} mutants have lost the ability to metabolize tropic acid.

Table 4.3
Pac and Php mutants of PMBL-1

Phenotype			Number of mutants per 10^4 colonies
Pac^{-}	Tro^{+}		0
Pac^{-}	Tro^{-}	Php^{+}	25
Pac^{-}	Tro^{-}	Php^{-}	4
Pac^{+}	Tro^{+}	Php^{-}	23

A mutant of the phenotype $Tro^{+}Pac^{-}$ has not been found, even not later in this research. This points to a role of phenylacetic acid in the tropic acid metabolism. These mutants have been subject of further study see 4.6.

The metabolism of p-hydroxyphenylacetic acid is much less related to that of tropic acid. Mutants with the phenotype $Tro^{+}Pac^{+}Php^{-}$ have been found. This makes it less plausible that p-hydroxyphenylacetic acid is an intermediate in the breakdown of tropic acid or phenylacetic acid.

4.6 INVESTIGATION OF MUTANTS BLOCKED IN THE METABOLISM OF PHENYLACETIC ACID

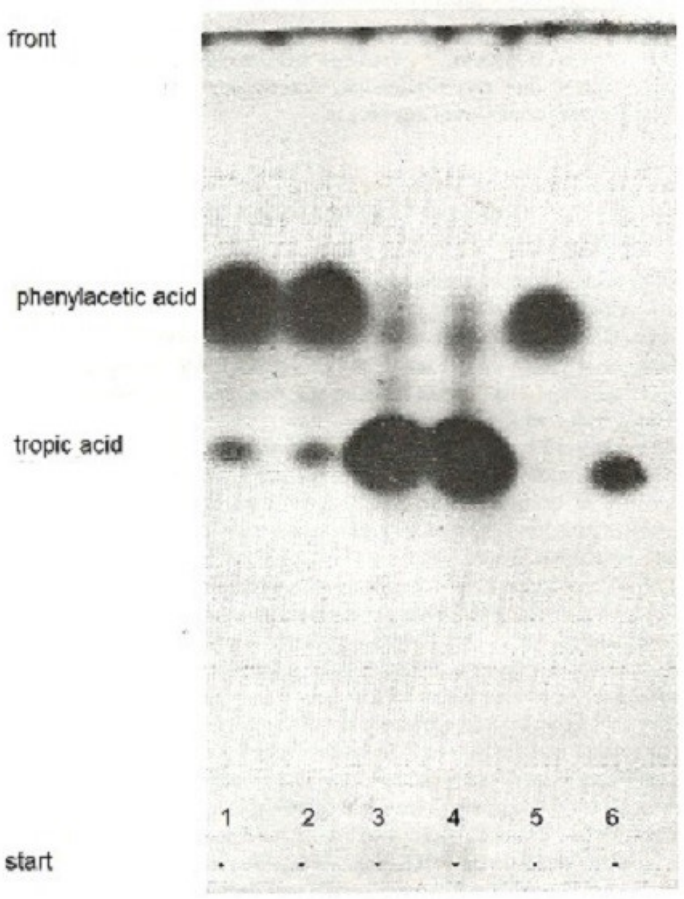
All mutants blocked in the metabolism of phenylacetic acid are no longer able to utilize tropic acid. The cultivation medium of these Pac^{-} mutants was analyzed in the search for the presence of tropic acid metabolites. The rationale for this experiment was the idea that the chance for a partially intact tropic acid metabolic pathway in Pac^{-} is larger than in mutants selected for the phenotype Tro^{-}.

The mutants were cultivated in a synthetic medium with 0.2 % tropic acid and 0.2% glucose as carbon source and shaken at 300 during 3 days. The bacteria were removed by centrifugation. The growth medium was acidified, extracted and analyzed by thin layer chromatography (chapter 2.7). The result for 2 Pac^{-} mutants (PMBL-114 and PMBL-107) is shown in fig 4.4. PMBL-114 has converted tropic acid nearly completely in a compound with the chromatographic behavior of phenylacetic acid. Under the same conditions PMBL-107 is able only to convert a small portion of the tropic acid. The conversion as found in the medium of PMBL-114 has been found later for 18 other Pac^{-} mutants.

In order to confirm the identity of the conversion product, the experiment with PMBL-114 has been repeated on a larger scale. The mutant was cultivated in 6 L synthetic medium containing in total 12 g tropic acid. The medium was subsequently treated as described above. About 5g of the converted product was obtained. The product recrystallized from petroleum ether 40-60^{0} C as tiny leaves. The compound was identified as phenylacetic acid: it behaves in thin layer chromatography as phenylacetic acid after elution with the solvents EMX and BEM. Its ultraviolet and infrared spectrum (Davies 1951) are identical to that of phenylacetic acid. In elementary analysis 70.75 % carbon and 6.01% hydrogen were found; in theory, one expects for phenylacetic acid 70.57% and 5.92%. The product isolated melts at 77.5^{0}, phenylacetic acid melts at 77,0^{0}, the melting point of the mixture is 77.0^{0}. These observations prove that tropic acid can be converted in phenylacetic acid by mutants of Pseudomonas PMBL-1.

Fig 4.4

Conversion of tropic acid in phenylacetic acid by pseudomonas mutants



Thin layer chromatogram of the extract of the cultivation medium of PMBL-114 (1 and 2) and PMBL-107 (3 and 4), cultivated in 0.2% glucose and 0.2% tropic acid during 3 days. Reference compounds phenyl acetic acid (5) and tropic acid (6). Elution fluid was EMX, detection by H_2SO_4-HNO_3 (1:1).

4.7 BREAKDOWN OF PHENYLACETIC ACID

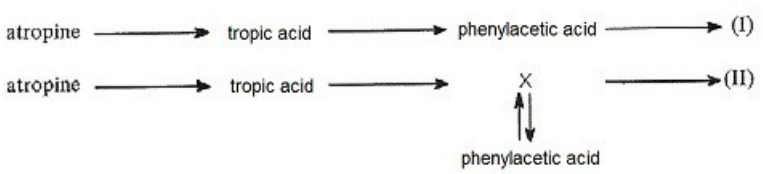
Phenylacetic acid is a rather normal carbon source for Pseudomonas (Stanier 1960). It is broken down by hydroxylation and oxygenation. Although the metabolism of phenylacetic acid in PMBL-1 has not been studied in detail, this study has provided some indications for the course of the breakdown of this compound. During the study of the mutants of the phenotype $Tro^{-}Pac^{-}$, one of these mutants (PMBL-112) appeared to convert tropic acid in a compound with an R_f value slightly higher than that of tropic acid.

Using preparative thin layer chromatography, a small amount of the compound was isolated in a chromatographically pure condition. The UV-spectrum showed the characteristic absorption of o-hydroxy- or m-hydroxyphenylacetic acid: a maximum at 271 nm and a shoulder at 277 nm. In further chromatography the o-hydroxy compound could be excluded. The compound with the higher R_f is therefore most probably m-hydroxyphenylacetic acid. The spectral and chromatographic data were not compatible with p-hydroxyphenylacetic acid neither with any of the other compounds in annex 1.

So probably, the breakdown of phenylacetic acid takes places through hydroxylation into m-hydoxyphenyl acetic acid.

4.8 DISCUSSION

Pseudomonas PMBL-1, cultivated in the presence of tropic acid is adapted to metabolize phenylacetic acid. Pseudomonas mutants can convert tropic acid in phenylacetic acid. Both findings confirm an intense metabolic relationship between these two aromatic acids. Nevertheless, one should not draw the immediate conclusion that phenylacetic acid is an intermediate in the breakdown of atropine and tropic acid (scheme1). It cannot be excluded that phenyl acetic acid is generated from the real intermediate X by a reversible reaction (scheme II) (Adelberg 1953).



In the breakdown according to scheme II, one expects to find mutants of the phenotype Pac^{-} Tro^{+}. However, all mutants selected for the phenotype Pac^{-} showed to be blocked in the breakdown of tropic acid. Mutagenic treatments

which resulted in the isolation of about 70 mutants with a specific mutation in the metabolism of atropine and tropic acid, did not yield mutants with the phenotype Pac^{-} Tro^{+}. Scheme II therefore is improbable. The conclusion is justified that the breakdown of atropine and tropic acid takes its course through a conversion into phenylacetic acid.

This conclusion is of major importance for the elucidation of the metabolic pathway of atropine and tropic acid and for the identification of the enzymes involved in this breakdown in Pseudomonas.