A MUTANT OF PSEUDOMONAS PUTIDA WITH ALTERED REGULATION OF THE ENZYMES FOR DEGRADATION OF PHENOL AND CRESOLS

G.J. WIGMORE AND R.C. BAYLY

DEPARTMENT OF MICROBIOLOGY,

MONASH UNIVERSITY MEDICAL SCHOOL,

COMMERCIAL ROAD, PRAHRAN, VICTORIA, AUSTRALIA

Received July 8,1974

SUMMARY

A mutant strain of Pseudomonas putida NCIB 10015 has been isolated that is unable to utilize para-cresol as a sole carbon source. The mutant strain grows very poorly when meta-cresol is to be utilized but grows rapidly at the expense of phenol or ortho-cresol. Preliminary evidence is presented that this mutant strain has a regulatory element of altered specificity. This regulatory element determines the induction of all known enzymes involved in the metabolism of phenol and ortho, meta and para-cresol. This is the first report of a mutation affecting the regulation of meta-cleavage enzymes.

INTRODUCTION

The regulation of the meta-cleavage pathway in P. putida NCIB 10015 has been studied by two different approaches. A phenol hydroxylase defective strain of P. putida was reported to be able to produce catechol 2,3-oxygenase, 2-hydroxymuconic semialdehyde hydrolase and 4-hydroxy-2-ketovalerate aldolase to levels similar to wild-type when grown in the presence of phenol (6). These results were confirmed by Sala-Trepat et al (12) who included 2-hydroxymuconic semialdehyde dehydrogenase, 4-oxolocrotonate tautomerase and 4-oxalocrotonate decarboxylase in their studies with the same mutant strain. These results demonstrated that phenol and not one of the intermediates of its degradation induced the whole suite of meta-cleavage enzymes.

A second approach has been to study the ratio of enzymes

formed under different conditions of induction (2, 6, 10). Although discrepancies in the ratios of enzymes induced can be demonstrated from the published data no author has been sufficiently confident to state whether or not they consider the enzymes of the meta-cleavage suite to be co-ordinately regulated in P. putida. Evidence that they were not co-ordinate in P. arvilla has been reported (7).

In this communication we report the isolation and some properties of a mutant strain that has lost the ability to induce all the meta-cleavage enzymes when grown in the presence of p-cresol. The mutant also has a reduced ability to induce these enzymes after growth in the presence of m-cresol. The properties of this mutant suggests that there is a regulatory element which controls the whole meta-cleavage suite of enzymes.

MATERIALS AND METHODS

The wild-type strain PsU-O, and the methods of maintenance, cultivation, induction, preparation of cell extracts and mutagenesis have been described (2). Mutant strain L2 was isolated using the penicillin/cycloserine technique with p-cresol as the contra-selective carbon source and o-cresol as the selective carbon source. Determination of oxygen uptakes and enzyme activities were by previously described methods:- catechol 2,3-oxygenase, hydrolase, aldehyde dehydrogenase (2), hydratase (3), aldolase (6) and tautomerase (11). The substrate for the tautomerase assay was formed in situ using phenol-induced extract of strain PsU-L1 which is tautomerase deficient (14).

A semi-quantitative estimation of decarboxylase activity

was made as follows: 2-hydroxymuconic semialdehyde (0.15 μmole) + NAD^+ (0.3 μmole) in 3 ml of M/10 phosphate buffer pH 7.4 was metabolized by a mixture of extracts of phenol-induced decarboxylase deficient strain PsU-L11 (14) and the strain to be tested. When the reaction was complete the reaction mixture was adjusted to pH 12 with 1ON NaOH and the absorbance at λ350 nm was determined. In the absence of test extract the increase in absorbance was approximately 0.8 and this was lowered in proportion to the decarboxylase activity of the extracts tested.

RESULTS

During selection of strains defective in the 4-oxalocrotonate region of the pathway (14) a mutant strain, L2, was isolated that on replication was unable to grow on m- and p-cresol but grew on phenol and o-cresol. The growth spectra was confirmed in liquid media and, with the exception of p-cresol and m-cresol where no growth occurred in 15 hours, the doubling times of wild-type and L2 were found to be the same when the phenolic compounds were used either as sole carbon source or in conjunction with fumarate. However, after incubation of L2 for 72 hours small colonies (0.5 mm diam.) were formed on m-cresol but there was no growth on p-cresol. Revertants of L2 which were selected by plating on p-cresol behaved as wild-type when grown on any of the phenolics. We found it impossible to distinguish revertants on m-cresol because of the slow growth of all the mutant cells plated.

When L2 was grown in the presence of m- or p-cresol no meta ring-fission products or the corresponding methylcatechols were detected. However, when L2 grew in the presence of phenol or o-cresol the strain behaved like wild-type (2).

TABLE 1 - Oxygen uptakes of wild-type O and mutant strain L2

Substrate	Inducers							
	phenol		o-cresol		m-cresol		p-cresol	
	O	L2	O	L2	O	L2	O	L2
phenol	154	143	56	60	70	12	46	<5
o-cresol	186	132	59	61	95	9	56	<5
m-cresol	144	113	24	42	46	8	29	<5
p-cresol	105	86	31	48	52	7	50	<5

Values, corrected for endogenous uptake, are expressed as μmole O_2/mg protein/hour.
In the absence of an inducer all values <5.

Therefore L2 does not have a hydroxylase enzyme which can convert either m- or p-cresol to their corresponding methylcatechol. This could be explained by the failure of these substrates to either enter the cell, induce the hydroxylase or serve as substrates for the hydroxylase. The change in specificity must occur in an event prior to the metabolism of the methylcatechols as mutant strain L2 responds differently to o- and m-cresol although both these compounds form 3-methylcatechol on hydroxylation (1).

All the phenolic compounds served as substrates for the phenol hydroxylase formed when L2 was induced by phenol or o-cresol and hence they must be able to enter the cells (Table 1) However those carbon sources which did not support growth were unable to induce a significant level of a hydroxylase active against any of the phenolic compounds. These results demonstrat that the behaviour of mutant strain L2 cannot be explained in terms of an altered substrate specificity of either the phenol hydroxylase or a specific phenolic transport system (if such an

activity exists). Therefore the lesion of L2 must involve a regulatory element.

The failure of m- and p-cresol to induce was not restricted to the hydroxylase as all known enzymes of the meta-cleavage pathway for phenol and cresols were similarly affected in mutant strain L2 (Table 2). In m-cresol-induced L2 there was about 10-20% of the level of enzymes demonstrated in m-cresol-induced wild-type. The low level of all the enzymatic activities reported (Tables 1 & 2) would be insufficient for growth of L2 in either liquid media or on replica-plating where 15 hour incubation periods were used.

DISCUSSION

In this preliminary communication we have described the isolation of a mutant which affects regulation of the meta-cleavage enzymes responsible for the metabolism of phenol and the cresols by *P. putida*.

The lesion does not appreciably alter the metabolism of phenol and o-cresol but affects m- and p-cresol dissimilation differently. This does not mean that there are different regulatory elements for the various phenolic compounds. It has been shown that apparently complete revertants selected on p-cresol are able to grow at the wild-type rate on m-cresol and hence a single lesion has affected differently the metabolism of m- and p-cresol. As an effect on a single element is different in response to m- and p-cresol it seems reasonable to assume that the same element can be involved in the metabolism of phenol and o-cresol without giving an altered response in the mutant.

Although the lesion must involve a regulatory element the pleiotrophy of the lesion can be accounted for in one of

TABLE 2 - Enzymatic activities of wild-type and mutant strain L2 after induction with phenol and cresols

Enzymes	PsU-O Inducer				PsU-L2 Inducer			
	phenol	o-cresol	m-cresol	p-cresol	phenol	o-cresol	m-cresol	p-cresol
Catechol 2,3-oxygenase[a]	0.14	0.09	0.12	0.06	0.17	0.10	0.02	<0.001
Hydrolase[a]	0.053	0.053	0.047	0.022	0.046	0.025	0.006	<0.001
NAD^+-dependent aldehyde dehydrogenase[a]	0.019	0.017	0.012	0.007	0.016	0.010	0.003	<0.001
Tautomerase[b,c]	1.0	1.1	0.8	0.6	1.1	0.8	0.2	0
Decarboxylase	++	++	++	++	++	++	±	-
Hydratase[a]	0.73	0.45	0.68	0.40	0.42	0.19	0.05	<0.01

a. Expressed as μmole of substrate formed/metabolized per minute per mg protein.
b. Expressed as units of activity where 1 unit = decrease of one absorbance unit at λ295nm per minute/mg protein.
c. Corrected for non-enzymatic breakdown of substrate.
Because of the low induced specific activities, comparative results for aldolase activity are not included. However, we have never been able to demonstrate a level of aldolase in p-cresol induced cells of mutant L2 which is greater than that found in uninduced cells.

two ways. Firstly, the lesion could affect the induction of a specific transport mechanism and therby limit the intra-cellular concentration of the effector so that induction of the enzymes of the meta-cleavage pathway is reduced. This "permease" could be part of the operon as occurs in the Lac operon (8) or map in a region distant from the genes for metabolic function as occurs in the L-ara system (5, 13). However no author has suggested that there is an active transport mechanism for phenolic compounds and where there are descriptions of transport mechanisms in aromatic degradation they normally function when the extracellular concentration of substrate is low (9). We favour the hypothesis that, under the experimental conditions used, no such transport mechanism is involved. If this is the case the regulatory element that is defective in strain L2 is one that determines the induction of all the enzymes involved in the meta-cleavage of phenolic compounds by the wild-type. The lesion is more likely to affect a regulatory protein than the site of action of such a regulatory protein. Models of repression and derepression involve an effector-induced allosteric change in a regulatory protein when the site on the DNA which recognizes the allosteric protein is able to distinguish the two forms of the regulatory protein. If it is considered that the lesion affects the recognition site it is necessary to infer that the effector plays a primary role in DNA recognition of the protein/effector complex. It seems more likely that the regulatory protein of mutant L2 has been altered so that p-cresol does not induce the allosteric change necessary for derepression and the ability of m-cresol in this respect is markedly reduced.

Thus we propose that the enzymes for meta-cleavage of phenolic compounds are under the control of a single regulatory protein, but we are unable to decide if this regulatory protein serves as a positive or negative control element. Although we have proposed a single regulatory protein we have not eliminated a model which involves the regulatory protein having more than one site of action, as has been described for example in glycerol metabolism (4) and arabinose degradation (5).

Further study of strain L2 and other regulatory mutant strains which we have isolated should resolve some of these questions.

ACKNOWLEDGEMENTS

The authors thank L. Johnson and D. Di Berardino for skilled technical assistance. The work was supported by an A.R.G.C. Grant No. D67/16545.

REFERENCES

1. Bayly, R.C., Dagley, S. and Gibson, D.T. (1966) Biochem. J. 101, 293-301.
2. Bayly, R.C. and Wigmore, G.J. (1973) J. Bacteriol. 113, 1112-1120.
3. Collinsworth, W.L., Chapman, P.J. and Dagley, S. (1973) J. Bacteriol. 113, 922-31.
4. Cozzarelli, N.R., Freedberg, W.B. and Lin, E.C.C. (1968) J. Mol. Biol. 31, 371-387.
5. Englesberg, E., Irr, J., Power, J. and Lee, N. (1965) J. Bacteriol. 90, 946-957.
6. Feist, C.F. and Hegeman, G.D. (1969) J. Bacteriol. 100, 869-877.
7. Feist, C.F. and Hegeman, G.D. (1969) J. Bacteriol. 100, 1121-1123.
8. Fox, C.F., Carter, J.R. and Kennedy, E.P. (1967) Proc. Nat. Acad. Sci., (Wash.) 57, 698-705.
9. Higgins, S.J. and Mandelstam, J. (1972) Biochem. J. 126, 917-922.
10. Murray, K. and Williams, P.A. (1974) J. Bacteriol. 117, 1153-1157.
11. Sala-Trepat, J.M. and Evans, W.C. (1971) Eur. J. Biochem. 20, 400-413.
12. Sala-Trepat, J.M., Murray, K. and Williams, P.A. (1972) Eur. J. Biochem. 28, 347-356.
13. Schleif, R. (1969) J. Mol. Biol. 46, 185-196.
14. Wigmore, G.J., Bayly, R.C. and Di Berardino, D. (1974) In Press.