SHORT COMMUNICATION

Evidence for the Existence of Two Catabolic Plasmids Coding for the Degradation of Naphthalene

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(Received 28 August 1979; revised 7 December 1979)

Two new and independently isolated catabolic plasmids coding for the degradation of naphthalene have been characterized in strains of *Pseudomonas putida*. Both plasmids are transmissible, belong to the P9 incompatibility group and code for naphthalene degradation via salicylate and catechol, then by the catechol *meta*-cleavage pathway.

INTRODUCTION

A transmissible plasmid encoding the degradation of naphthalene via the catechol *meta*-cleavage pathway was reported by Dunn & Gunsalus (1973). Boronin *et al.* (1976) presented evidence for another plasmid system which encoded the enzymes involved in the conversion of naphthalene to salicylate. The present paper reports the identification of two new and independently isolated NAH catabolic plasmids.

METHODS

The bacterial strains, plasmids and bacteriophage used are listed in Table 1. General procedures have been described elsewhere (Austen & Dunn, 1977a; White & Dunn, 1977). Specific references to techniques used are as follows: plasmid transfer by transduction (White & Dunn, 1977); mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Fargie & Holloway, 1965); plasmid transfer by conjugation, growth of organisms for enzyme assay, preparation of extracts and assay of catechol 2,3-dioxygenase (EC 1.13.11.2) (Austen & Dunn, 1977a, 1980). One unit of activity of catechol 2,3-dioxygenase is defined as that which oxidizes 1.0 μmol catechol min^{-1} . The growth media consisted of salts medium PAS (Chakrabarty, 1972) supplemented with the appropriate carbon sources.

RESULTS AND DISCUSSION

Two naphthalene-utilizing bacteria were isolated from the Sydney area. One strain [UB1-1(pND140)] was obtained from a garden soil sample where naphthalene flakes had been intermittently added for at least 2 years to deter animals from digging. The other strain [UB2-1(pND160)] was obtained from a soil sample where there had been no apparent enrichment for naphthalene utilization. Since later experiments provided evidence that both strains harboured plasmids, they will be referred to in this way from the outset. The origins of the samples from which UB1-1(pND140) and UB2-1(pND160) were obtained were approximately 5 miles apart and the colony morphologies of the strains were markedly different.

Table 1. *Bacterial strains, plasmids and bacteriophage*

<i>Bacterial strains*</i>		
PP1-2	wild-type	Wong & Dunn (1974)
PP1-3	<i>ben-1</i>	Wong & Dunn (1974)
PP1-8	<i>met-1</i>	Wong & Dunn (1974)
PP1-10	<i>ben-2</i>	Austen & Dunn (1977 <i>a</i>)
PP1-18	<i>ben-1 phl-1</i>	Wong & Dunn (1976)
PP1-25(CAM)	<i>trp-1</i> (CA M)	Hewetson <i>et al.</i> (1978)
PAr1-6(TOL)	<i>met-1 tra-1</i> (TOL)	Wong & Dunn (1976)
UB1-1(pND140)	wild-type	Soil isolate
UB1-2(pND140)	<i>trp-1</i>	MNNG mutagenesis of UB1-1(pND140)
UB2-1(pND160)	wild-type	Soil isolate
UB2-2(pND160)	<i>met-1</i>	MNNG mutagenesis of UB2-1(pND160)
<i>Catabolic plasmids</i>		
TOL	Williams & Murray (1974); Wong & Dunn (1974)	
CAM	Rheinwald <i>et al.</i> (1973)	
NAH	Dunn & Gunsalus (1973)	
pND140	New NAH plasmid	
pND160	New NAH plasmid	
<i>Bacteriophage</i>		
pf16	Gunsalus <i>et al.</i> (1968)	

* *ben*, requirement for benzoate; *met*, requirement for methionine; *phl*, requirement for phenol; *trp*, requirement for tryptophan; *tra*, increased transfer frequency of resident plasmid. MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The prefixes UB1 and UB2 refer to unidentified bacteria no. 1 and 2.

Transfer of plasmids pND140 and pND160 to defined strains of *Pseudomonas putida* was achieved following the isolation of UB1-2(pND140), a tryptophan-requiring auxotroph of UB1-1(pND140), and UB2-2(pND160), a methionine-requiring auxotroph of UB2-1(pND160). Using the auxotrophs as donors, plasmids pND140 and pND160 were transferred to the defined bacterial strains PP1-2, PP1-3, PP1-10 and PP1-18 using the plate mating method. Selection of transconjugants was achieved on PAS plus naphthalene plates; each plasmid was transferred at a frequency of approximately 10^{-6} per donor cell. Growth responses were tested on a variety of carbon sources in order to determine the catabolic properties coded for by pND140 and pND160 (Table 2). Results for various defined strains harbouring the original NAH plasmid (Austen & Dunn, 1977*a*) are included for comparison. Strain PP1-2 is able to grow on benzoate or phenol which are converted to catechol and then further catabolized by the *ortho*-cleavage pathway. In this host strain *m*-toluate can be converted to 3-methylcatechol but is not catabolized further. Accumulated 3-methylcatechol is oxidized chemically and the medium turns black. Introduction of plasmids NAH, pND140 and pND160 into PP1-2 is accompanied by the appearance of phenotypes for growth on naphthalene, salicylaldehyde and salicylate. The presence of the NAH plasmid permits slow growth on *m*-toluate. This arises because *m*-toluate can be converted to 3-methylcatechol by the host cell and *m*-toluate causes partial induction of the NAH-encoded *meta*-cleavage pathway (Austen & Dunn, 1977*b*). Neither pND140 nor pND160 seem able to allow this growth response. In the absence of a functional *ortho*-cleavage pathway encoded by the host cell, *P. putida* PP1-3 harbouring the plasmids is unable to utilize benzoate or phenol. The black coloration arises because these compounds give rise to the accumulation of catechol which is then oxidized chemically. For *P. putida* PP1-3 harbouring pND140 and pND160, large colonies appeared on benzoate and phenol plates and these were shown to be regulatory mutants in the plasmid-encoded catechol *meta*-cleavage pathway (Austen & Dunn, 1980). To obtain the large colonies it is essential that the host cell has a functional system for converting benzoate or phenol to catechol. This

Table 2. *Growth responses of P. putida strains derived from PP1-2 harbouring NAH, pND140 and pND160*

The defined host strains have the following properties: PP1-2 codes for conversion of benzoate and phenol to catechol after which catechol is degraded by the *ortho*-cleavage pathway; PP1-3 is a mutant of PP1-2 defective in catechol 1,2-dioxygenase, the first enzyme of the *ortho*-cleavage pathway; PP1-18 is a mutant of PP1-3 defective in phenol 2-monooxygenase which catalyses the conversion of phenol to catechol; PP1-10 is a mutant of PP1-2 defective in the conversion of benzoate to catechol.

Incubation was for 3 d at 30 °C. Responses were rated as follows: 4+, colonies about 2 mm diam.; 3+, 1 mm; 2+, 0.5 mm; +, 0.1 mm; —, no growth. L indicates that large colonies, at least 0.5 mm diam., appeared after 2 d incubation at a frequency of about 10^{-7} for strains harbouring pND140 and pND160 and after 5 d incubation for strains harbouring NAH. B indicates a black or brown coloration of the medium after about 2 d incubation.

Bacterial and plasmid strain	Growth response on substrate						
	Naphthalene (vapour phase)	Salicyl-aldehyde (2.5 mm)	Salicylate (10 mm)	Benzoate (10 mm)	<i>m</i> -Toluate (10 mm)	<i>p</i> -Toluate (10 mm)	Phenol (2.5 mm)
PP1-2	—	—	—	4+	—(B)	—	3+
PP1-2(NAH)	4+	+	4+	4+	+	—	3+
PP1-2(pND140)	4+	+	4+	4+	—(B)	—	3+
PP1-2(pND160)	4+	+	4+	4+	—(B)	—	3+
PP1-3	—	—	—	—(B)	—(B)	—	—(B)
PP1-3(NAH)	4+	+	4+	—(B)	+	—	—(B,L)
PP1-3(pND140)	4+	+	4+	—(B,L)	—(B)	—	—(B,L)
PP1-3(pND160)	4+	+	4+	—(B,L)	—(B)	—	—(B,L)
PP1-10	—	—	—	—	—	—	3+
PP1-10(NAH)	4+	+	4+	—	—	—	3+
PP1-10(pND140)	4+	+	4+	—	—	—	3+
PP1-10(pND160)	4+	+	4+	—	—	—	3+
PP1-18	—	—	—	—(B)	—(B)	—	—
PP1-18(NAH)	4+	+	4+	—(B)	+	—	—
PP1-18(pND140)	4+	+	4+	—(B,L)	—(B)	—	—
PP1-18(pND160)	4+	+	4+	—(B,L)	—(B)	—	—

indicates a composite growth phenotype, part arising from the host cell and part from the resident plasmid. Similar regulatory mutants and the composite growth phenotype have been reported previously for NAH (Austen & Dunn, 1977*a, b*). The appearance of large colonies on benzoate for *P. putida* strains PP1-3 and PP1-18 harbouring plasmids pND140 and pND160 suggests that these systems are less sensitive to the toxic effects arising from the chemical oxidation of catechol when compared to strains PP1-3 or PP1-18 harbouring NAH.

To verify that pND140 and pND160 encode the *meta*-cleavage pathway, catechol 2,3-dioxygenase, which is the first enzyme of the *meta*-cleavage pathway, was assayed. The specific activities in extracts of PP1-10(pND140) and PP1-10(pND160) were estimated following growth on PAS plus 15 mM-acetate and on PAS plus 10 mM-acetate plus 5 mM-salicylate. Specific activities of 35, 30, 290 and 280 munits (mg protein)⁻¹, respectively, were obtained. Growth in the presence of salicylate resulted in induction of catechol 2,3-dioxygenase encoded by both pND140 and pND160. The pH optimum for each enzyme was found to be 7.2 compared to the value of 8.3 for the enzyme encoded by the original NAH plasmid (Austen & Dunn, 1977*a*). The host strain has not been demonstrated to code for catechol 2,3-dioxygenase (Austen & Dunn, 1977*b*).

A phage preparation of pf16 grown on PP1-2(pND140) and PP1-2(pND160) was used as the transducing preparation and selection was made for transduction of the ability to utilize salicylate with PP1-8 as recipient. Transductants were obtained from each preparation at a frequency of approximately 10^{-9} per phage particle. Two transductants from each experiment were tested and were found to utilize naphthalene, to retain transmissibility

and to encode catechol 2,3-dioxygenase. This suggests close linkage of the genetic markers encoding catabolic properties with those for transfer properties.

Plasmids which belong to the P2 and P9 incompatibility groups can be identified by introduction of TOL (P9) and CAM (P2) into the host strain. TOL and CAM were transferred from PAR1-6(TOL) and PP1-25(CAM), respectively, to PP1-2(pND140) and PP1-2-(pND160) using *p*-toluate and camphor selection, respectively, and auxotrophic counterselection. TOL and CAM were also transferred to PP1-2 to determine if pND140 and pND160 residence in PP1-2 limited entry of TOL or CAM. TOL transferred to PP1-2, PP1-2-(pND140) and PP1-2(pND160) at a frequency of approximately 10^{-4} transconjugants per donor cell. CAM transferred to the same three strains at approximately 10^{-2} transconjugants per donor cell. Therefore pND140 and pND160 did not limit TOL or CAM entry to PP1-2.

Sixteen transconjugants from each conjugation were purified by single colony isolation and grown through approximately 75 generations on the respective selection medium. Tests for growth on naphthalene of two single colonies from each of the purified transconjugants indicated that all of the CAM transconjugants but none of the TOL transconjugants tested retained pND140 or pND160. To verify that the presence of TOL was not interfering with expression of pND140 and pND160, strains were obtained from which TOL had segregated spontaneously. All segregants remained unable to utilize naphthalene or salicylate. These results suggest that pND140 and pND160 belong to the same incompatibility group as TOL and the original NAH plasmid, that is the P9 incompatibility group.

The two new plasmids, pND140 and pND160, appear identical with each other in all tests that were applied in this limited study. Three differences were detected between these plasmids and NAH when hosted by PP1-2 and its mutant derivatives. Strains harbouring the new plasmids were unable to grow on *m*-toluate despite host-specified conversion of this substrate to 3-methylcatechol; under the induction conditions used this was found to be due in part to lack of induction of the plasmid-borne *meta*-cleavage pathway (Austen & Dunn, 1980). Apparent regulatory mutants (large colonies) could be readily isolated on benzoate medium and the pH optimum for catechol 2,3-dioxygenase encoded by pND140 and pND160 differed from that encoded by NAH.

It is interesting that both new plasmids belong to the P9 plasmid incompatibility group which contains TOL and NAH (White & Dunn, 1978), SAL (Jacoby, 1979) and a *p*-cresol-utilizing plasmid pND50 (Hewetson *et al.*, 1978). A physical study to determine the genetic similarity between all these plasmids would be very rewarding. Detailed determination of the relationship between the different naphthalene catabolic pathways may also provide useful information on their evolutionary origin. This should include studies on those pathways known to be plasmid-borne and some for which no evidence has been obtained to implicate plasmids.

This work was supported by a grant from the Australian Research Grants Committee. R. A. A. was supported by an Australian Commonwealth Postgraduate Scholarship and H. M. D. by the A.R.G.C. grant. The strains UB1-1(pND140) and UB2-1(pND160) were isolated in a student practical class co-ordinated by Dr P. Rowntree, Microbiology Department, University of New South Wales.

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