Isolation, Characterization and Mapping of Mandelate Pathway Mutants of *Acinetobacter calcoaceticus*

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Mutants of *Acinetobacter calcoaceticus* EBF 65/65 that could not grow on intermediates of the mandelate or benzyl alcohol pathways were isolated and in some cases the enzymic lesions were identified. Several catabolic markers were mapped using the plasmid pAV1. The mandelate genes appeared to be clustered near the auxotrophic marker *phe-1* but were not all contiguous with each other. The gene responsible for the appearance of the novel L(+)-mandelate dehydrogenase appeared to be close to a gene responsible for the activity of the original D(–)-mandelate dehydrogenase.

INTRODUCTION

A number of bacteria and fungi can metabolize mandelate but the precise nature of the enzymes and of the regulation of their expression varies from organism to organism (Bhat & Vaidyanathan, 1976; Kenyon & Hegeman, 1979). *Acinetobacter calcoaceticus* wild-type strain EBF 65/65 can grow on D(–)-mandelate and some mutants with a novel L(+)-mandelate dehydrogenase can also grow on L(+)-mandelate (Hills & Fewson, 1983*a, b*). In addition, *A. calcoaceticus* metabolizes benzyl alcohol by a pathway which converges with that for the oxidation of mandelate (Fig. 1).

A start has been made on mapping some of the genes involved in mandelate metabolism by pseudomonads. In *Pseudomonas putida* the genes encoding the first five enzymes of the mandelate pathway seem to be linked and may even be contiguous (Chakrabarty *et al.*, 1968; Chakrabarty & Gunsalus, 1969; Wheelis & Stanier, 1970; Wheelis, 1975; Hegeman & Root, 1976). These genes show supra-operonic clustering with genes of functionally related pathways (Wheelis & Stanier, 1970; Leidigh & Wheelis, 1973). The situation in *P. aeruginosa* is rather different. Whilst there is some clustering of genes for related pathways, two of the mandelate genes are separated by a group of genes encoding enzymes for the degradation of benzoate and catechol (Kemp & Hegeman, 1968; Rosenberg & Hegeman, 1969).

The aims of the present work were threefold. First, to obtain mutants of *A. calcoaceticus* that would be useful in physiological and biochemical studies. Secondly, to obtain some information about the genetic organization of the mandelate pathway to allow comparison with the situation in pseudomonads. Thirdly, to see whether the gene responsible for the appearance of the novel L(+)-mandelate dehydrogenase activity mapped close to that of the original D(–)-mandelate dehydrogenase; these two enzymes have very similar properties and are co-ordinately regulated (Hills & Fewson, 1983*a, b*). Some mutants of *A. calcoaceticus* wild-type strain NCIB 8250 have been obtained (Livingstone & Fewson, 1972; Fewson *et al.*, 1978; Hills & Fewson, 1983*a, b*), but unfortunately there is no genetic mapping system available for this strain. We therefore

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Fig. 1. Metabolism of mandelate and benzyl alcohol by *A. calcoaceticus* EBF 65/65. The wild-type strain grows on only the D(-)-isomer of mandelate but some mutants have a novel L(+)-mandelate dehydrogenase (1) and can grow on the L(+)-isomer (Hills & Fewson, 1983*b*). D-Mandelate dehydrogenase (2), L(+)-mandelate dehydrogenase (when present), phenylglyoxylate decarboxylase (3) and benzaldehyde dehydrogenase I (5) are co-ordinately induced by phenylglyoxylate and are designated the regulon-1 enzymes. Benzyl alcohol dehydrogenase (4) and benzaldehyde dehydrogenase II (6) are co-ordinately induced by benzaldehyde or benzyl alcohol and are designated the regulon-2 enzymes. Further details were given by Hills & Fewson (1983*b*).

embarked on experiments with the wild-type strain EBF 65/65 because it has a mandelate pathway which is very similar to that in strain NCIB 8250 (Hills & Fewson, 1983*b*) and about 23 of its chromosomal genes have been mapped using the plasmid RP4 (Towner & Vivian, 1976*a*; Towner, 1978). Plasmid pAV1, which specifies resistance to sulphonamides, was later shown to mobilize the chromosome at a higher frequency (Hinchliffe & Vivian, 1980*b*) and this has been used for mapping in the present work.

METHODS

Growth of bacteria. Strains of *A. calcoaceticus* are listed in Table 1. *Escherichia coli* C600 (pRP14⁺) (RP4::Tn7; Ap^RKm^RTc^RTp^RSm^R; Barth & Grinter, 1977) was obtained from Dr P. T. Barth, ICI Corporate Laboratory, Runcorn, Cheshire, UK.

Minimal medium, tryptone soya agar and nutrient broth were as described by Towner & Vivian (1976*a*) and salts medium as described by Hills & Fewson (1983*b*).

A. calcoaceticus was grown at 23 °C for the measurement of enzyme activities and at 28 °C in all other experiments; *E. coli* was grown at 37 °C.

Sulphadiazine (Sigma) and trimethoprim lactate (Burroughs Wellcome) were used at 500 µg ml⁻¹ and tetracycline.HCl (Sigma) at 5 µg ml⁻¹.

Mutagenesis. EMS (Sigma) was added to an overnight nutrient broth culture to give a final concentration of 2% (w/v) and subsequently the culture was incubated on a rotary shaker for 2 h. The bacteria were centrifuged and the pellet washed in quarter-strength Oxoid Ringer's solution to remove any traces of EMS and resuspended in 5 ml nutrient broth; the suspension was then incubated on a rotary shaker for 4 h. A suitable dilution of the culture was then spread on tryptone soya agar to give about 100 colonies per plate. After 48 h incubation, the bacteria were replica plated on selective media. Potential catabolic mutants were plated on salts medium solidified with 1.5% (w/v) Difco Bacto Agar and containing appropriate auxotrophic supplements and carbon sources, in the following order: benzyl alcohol (5 mM), D(-)- or L(+)-mandelate (5 mM), phenylglyoxylate (5 mM), benzoate (2 mM). Plates were examined after incubation for 48 h.

Filter matings and analysis of recombinants. Conjugative plasmid pAV1 (Hinchliffe & Vivian, 1980*a*) was used in the method described by Towner & Vivian (1976*a*). Recombinants were analysed for co-inheritance as described by Towner (1978).

Table 1. *Strains of A. calcoaceticus*

All strains were derived from wild-type EBF 65/65 (Mdd^o Mdl^o Pgc^o Bad^o) and unless stated otherwise harboured pAV2 (Hinchliffe *et al.*, 1980).

Strain	Characteristics*	Reference or source
EBF 65/65	Wild-type	Towner & Vivian (1976a)
C48	<i>ile-1 met-1</i>	Towner & Vivian (1976b)
C426	<i>his-1 trp-2</i>	Hinchliffe & Vivian (1980b)
C484	<i>phe-1 thi-2</i>	Hinchliffe <i>et al.</i> (1980)
C4103	<i>leu-2 phe-1 thi-2</i>	EMS treatment of C484
C4151	<i>his-1 trp-2</i> pAV1 ⁺	Hinchliffe & Vivian (1980b)
C4161	<i>phe-1 thi-2</i> pAV2 ⁻	Hinchliffe <i>et al.</i> (1980)
C4183	<i>cys-3 phe-1 mdd-1 pgc-1</i>	Cross DV45, this paper
C4185	<i>ala-1 cys-3</i> pAV1 ⁺	Cross DV6 (Vakeria, 1982): C4151 × C459 (<i>ala-1 cys-3</i>)
C4201	<i>ile-1 met-1</i> Bad ^{no}	EMS treatment of C48
C4205	<i>ile-1 met-1</i> Bad ^{no}	EMS treatment of C48
C4207	<i>ile-1 met-1</i> Bad ^{no}	EMS treatment of C48
C4223	<i>phe-1 thi-2 mdd-1 pgc-1</i> pAV2 ⁻	Spontaneous mutant of C4161
C4226	<i>ile-1 met-1 mdd-2</i>	EMS treatment of C48
C4248	<i>ile-1 met-1 mdl-1</i>	Hills & Fewson (1983b)
C4267	<i>ala-1 met-1 mdd-2</i> pAV1 ⁺	Cross DV32 (Vakeria, 1982): C4185 × C4226
C4279	<i>cys-3 trp-2</i> pAV1 ⁺	Cross DV28 (Vakeria, 1982): C4185 × C4275 (<i>met-1 trp-2</i>)
C4289	<i>met-1 phe-1 mdd-2</i>	Cross DV38, this paper
C4290	<i>met-1 phe-1 mdd-1 pgc-1</i>	Cross DV39 (Vakeria, 1982): C4266 (<i>his-1 phe-1 mdd-1 pgc-1</i> pAV1 ⁺) × C4275
C4295	<i>met-1 phe-1 mdd-2 mdl-2</i>	Spontaneous mutant of C4289
C4408	<i>ala-1 his-1 leu-2 mdl-2</i> pAV1 ⁺	Cross DV52 (Vakeria, 1982): C4287 (<i>ala-1 his-1 leu-2 trp-2</i> pAV1 ⁺) × C4295
C4413	<i>mdd-3</i>	EBF 65/65; this paper
C4420	<i>ile-1 met-1</i> Bad ^{no} Mdd ^{no} Asa ⁺ Hg ⁺	C4205 (Vakeria, 1982)
C4435	<i>met-1 trp-2 mdd-3</i> pAV1 ⁺	} Cross DV219a (D. Vakeria, unpublished): C4426 (<i>ala-1 his-1 met-1 trp-2 rif^R</i> pAV1 ⁺) × C4413
C4437	<i>his-1 met-1 trp-2 mdd-3</i> pAV1 ⁺	
C4438	<i>ala-1 his-1 trp-2 mdd-3</i> pAV1 ⁺	
C4439	<i>ile-1 met-1 mdl-1</i> pAV1 ⁺	
C4440	<i>phe-1 thi-2</i> pAV1 ⁺	Cross DV221 (D. Vakeria, unpublished): C4434 (<i>phe-1 thi-2</i> pAV2 ⁻ pAV1 ⁺) × C4248
C4441	<i>ile-1 met-1 mdc-1</i> pAV1 ⁺	Cross DV224, this paper Cross DV223 (D. Vakeria, unpublished): C4434 × C4664
C4664	<i>ile-1 met-1 mdc-1</i>	Hills & Fewson (1983b)

* Genotype designations are standard (Demerec *et al.*, 1966; Novick *et al.*, 1976) except for: *mdc*, constitutive for the regulon-1 enzymes; *mdd*, no growth on D(-)-mandelate; *mdl*, growth on L(+)-mandelate; *pgc*, no growth on phenylglyoxylate. For phenotypes the superscripts 'g' and 'ng' indicate 'growth' or 'no growth' respectively on: Mdd, D(-)-mandelate; Mdl, L(+)-mandelate; Pgc, phenylglyoxylate; Bad, benzyl alcohol or benzoate.

Use of transposon Tn7. Filter mating between *A. calcoaceticus* EBF 65/65 and *E. coli* C600 was done as outlined above. The bacteria were plated on minimal medium containing tetracycline and trimethoprim. A single colony was inoculated into nutrient broth containing trimethoprim and incubated at 4 °C for 14 d. The bacteria grew at this temperature and the resulting culture was then diluted and spread on tryptone soya agar; after 48 h incubation at 28 °C the resulting colonies were replica plated to test for resistance to tetracycline and trimethoprim and for the ability to grow on D(-)-mandelate, phenylglyoxylate, benzyl alcohol or benzoate.

Measurement of enzyme activities. Bacteria were grown and harvested as described by Hills & Fewson (1983 b) except that the inoculum was 12.5% (v/v) and the cultures (80 ml in 250 ml conical flasks) were incubated on a rotary shaker before centrifugation. Activities of L(+)- and D(-)-mandelate dehydrogenases, phenylglyoxylate (benzoylformate) decarboxylase (EC 4.1.1.7) and benzyl alcohol dehydrogenase were measured as summarized by Hills & Fewson (1983b). Activities of the two benzaldehyde dehydrogenases were estimated separately on the basis of their different heat stabilities (Livingstone *et al.*, 1972). Extracts prepared by ultrasonic treatment (Hills & Fewson, 1983b) were diluted with an equal volume of 0.1 M-sodium pyrophosphate buffer, pH 9.5, and assayed immediately using the procedure of Beggs & Fewson (1977) (benzaldehyde dehydrogenase I + II) and then after 4 h incubation at 37 °C (benzaldehyde dehydrogenase I); the activity of benzaldehyde dehydrogenase II was

estimated as the difference between the two rates. This procedure is more convenient than that used previously (Livingstone *et al.*, 1972) and its reliability was confirmed by measuring the enzyme activities of mixtures of extracts of bacteria that had been grown in the presence of mandelate, thiophenoxyacetate or benzyl alcohol. Catechol 2,3-oxygenase (EC 1.13.11.2) activity was measured by the spectrophotometric procedure of Sala-Trepat & Evans (1971) and catechol 1,2-oxygenase (EC 1.13.11.1) by the method of Hegeman (1966). All mutants were grown and assayed at least twice; representative results are given in the text.

RESULTS AND DISCUSSION

Isolation of mutants and their characterization

Mutants which contained a novel L(+)-mandelate dehydrogenase or which were constitutive for the mandelate enzymes could be isolated without difficulty from a range of parental strains using established positive selection techniques (Hills & Fewson, 1983*a, b*) but blocked mutants were more difficult to obtain. More than 10⁵ colonies of wild-type strain EBF 65/65 and auxotrophic strains C48, C426 and C4161 were screened in attempts to isolate mutants lacking one or more enzymes of the mandelate or benzyl alcohol pathways. Several enrichment and selection techniques were used, including some that were moderately successful with wild-type strain NCIB 8250 (e.g. Livingstone *et al.*, 1972; Ahlquist *et al.*, 1975). Mutagenesis with EMS was used in some experiments. Only 43 mutants were obtained which could not grow on one or more intermediates of the pathways. Some of these mutants proved to be unstable but others (Tables 1 to 4) were suitable for mapping work.

No stable mutants were obtained in which the activity of benzyl alcohol dehydrogenase and/or benzaldehyde dehydrogenase II was diminished; this was particularly unfortunate because it may be necessary to delete benzaldehyde dehydrogenase II activity before attempting to isolate mutants lacking benzaldehyde dehydrogenase I. Surprisingly, some mutants (e.g. C4201) which could not grow on benzyl alcohol contained elevated activities of the regulon-2 enzymes (Tables 2 and 3). Crosses using mutants of this sort sometimes gave rise to unexpected phenotypes in the progeny. Thus, in a cross involving C4151 and C4201, both parents could grow on D(-)-mandelate but when 201 recombinants from the four auxotrophic selections were tested, one was found to be unable to grow on either phenylglyoxylate or D(-)-mandelate. Further unexpected recombinants, including some that were Mdd^{no} Pgc^{no} and others that were Bad^{no} Mdd^{no} Pgc^{no}, were isolated from crosses in which the parents were Bad^{no} Mdd^{no} Pgc^{no}/Bad⁺ Mdd⁺ Pgc⁺ and Bad⁺ Mdd⁺ Mdl^{no} Pgc^{no}/Bad⁺ Mdd⁺ Mdl⁺ Pgc⁺ respectively. Crosses involving strains which were unaltered in their catabolic activities with respect to the wild-type strain failed to yield unexpected phenotypes, indicating that segregants unable to grow on certain aromatic substrates arose only in crosses involving strains already altered in their catabolic abilities. These observations indicate that there may be some sort of genetic interaction between regulons 1 and 2.

Mutant C4413 (mdd-3). An RP4 derivative carrying transposon Tn7, pRP14 (RP4::Tn7; Barth & Grinter, 1977), was used in an attempt to isolate catabolic mutants. Strain EBF 65/65 can grow at 4 °C, but replication of RP4 appears to be hindered at this temperature (Errington, 1981). The introduction of pRP14 into strain EBF 65/65 followed by growth at 4 °C and selection for trimethoprim resistance could result in the transposition of Tn7 into the bacterial chromosome or into another plasmid. Only one variant strain was isolated by this procedure; this was C4413, which failed to grow on D(-)-mandelate but showed wild-type behaviour towards the other substrates tested (Table 2) and lacked D(-)-mandelate dehydrogenase activity (Table 3). The pattern of enzyme activity is consistent with phenylglyoxylate, but not mandelate, being the inducer of the regulon-1 enzymes; this specificity is the same as that found in wild-type strain NCIB 8250 (Livingstone & Fewson, 1972). At first, the strain was resistant to both trimethoprim (indicative of Tn7) and tetracycline (indicative of RP4), and the presence of pRP14 was confirmed by agarose gel electrophoresis. Wild-type strain EBF 65/65 has been shown to harbour a plasmid pAV2 which mediates a restriction and modification system influencing the inheritance of pAV1 (Hinchliffe *et al.*, 1980). In mutant C4413, the band corresponding to pAV2 had increased in size by approximately 1 to 2 MDal. With repeated

Table 2. Growth of strains of *A. calcoaceticus* on various intermediates of the mandelate and benzyl alcohol pathways

Growth was tested on salts medium solidified with 1.5% (w/v) Difco Bacto Agar containing appropriate auxotrophic supplements. The substrates were tested at 5 mM, except for benzoate which was used at 2 mM. +, Growth; -, no growth.

Strain	Carbon source	Growth				
		L(+)-Mandelate	D(-)-Mandelate	Phenylglyoxylate	Benzyl alcohol	Benzoate
Wild-type EBF 65/65		-	+	+	+	+
C4201		-	+	+	-	+
C4223		-	-	-	+	+
C4226		-	-	+	+	+
C4295		+	-	+	+	+
C4413		-	-	+	+	+

subculturing, mutant C4413 gradually lost pRP14 and became sensitive to both trimethoprim and tetracycline; however, the Mdd^{ns} phenotype and the apparent insertion in pAV2 were retained.

We have not been able to determine whether mutant C4413 arose as a direct effect of transposition, as a result of some indirect effect of Tn7 or by some other mechanism.

Mutant C4223 (*mdd-1 pgc-1*). This mutant was isolated as a spontaneous variant of strain C4161 and was unable to grow on D(-)-mandelate or phenylglyoxylate (Table 2). Activities of D(-)-mandelate dehydrogenase, phenylglyoxylate decarboxylase and benzaldehyde dehydrogenase I were all absent (Table 3) and so this may be a regulatory mutant. Unlike the situation in the parental strain C4161 or the wild-type EBF 65/65, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II (the regulon-2 enzymes) were induced in the presence of phenylglyoxylate, the inducer of the regulon-1 enzymes (Table 3). Similar behaviour was observed for certain mandelate pathway mutants of *A. calcoaceticus* wild-type strain NCIB 8250 (Livingstone & Fewson, 1972). There is no clear explanation of this interaction between the two regulons; however, it is reminiscent of the observation that the regulon-2 enzymes cannot be induced in mutants constitutive for the mandelate enzymes (Beggs & Fewson, 1977; Fewson *et al.*, 1978; Hills & Fewson, 1983b). It appears that there may be some sort of reciprocal expression of the two systems and this in turn may be connected with the unexpected isolation of Mdd^{ns} recombinants from crosses involving Bad^{ns} Mdd^s parents described above.

The spontaneous occurrence of mutant C4223 was unexpected because no spontaneous Mdd^{ns} variants were obtained with wild-type strain EBF 65/65 or auxotrophic mutants C48, C426 or C484. However, all these strains contained pAV2 whereas mutant C4161 lacked this plasmid. Subsequent preliminary experiments have shown that storage at room temperature of cultures of strains lacking pAV2 revealed a certain degree of phenotypic instability and Mdd^{ns} segregants were readily isolated; some of these gradually reverted over 3 to 4 months but C4223 has remained stable (Table 4).

Mutant C4226 (*mdd-2*). Several putative mutants blocked in the mandelate pathway were difficult to isolate as pure strains because on repeated subculturing they showed continuous sectoring of Mdd^s and Mdd^{ns} zones within the colonies. Mutant C4226 was successfully isolated from the auxotrophic strain C48, but it did give Mdd^s revertants at low frequency (Table 4) and these showed consistent changes in the plasmid profile of the strains, involving an increase in size of about 7 MDal in a large cryptic plasmid as well as the appearance of a novel plasmid band of about 14 MDal (D. Vakeria & A. Vivian, unpublished observations). Strain C4226 did not grow on D(-)-mandelate (Table 2), presumably because of failure to induce sufficient D(-)-mandelate dehydrogenase activity in the presence of D(-)-mandelate (Table 3). It had about half the D(-)-mandelate dehydrogenase activity of the wild-type strain when grown in the presence

Table 3. *Enzyme activities of strains of A. calcoaceticus*

Bacteria were grown in 5 mM-L-glutamate/salts medium, containing appropriate amino acids in the case of auxotrophic strains. The additions were at 5 mM, except for benzaldehyde which was 1.5 mM. Under all conditions except those marked * there was catechol 1,2-oxygenase activity [150 to 350 nmol min⁻¹ (mg protein)⁻¹] present; catechol 2,3-oxygenase activity was never detected, even after growth on phenol. Experiments in which extracts were treated with 30 mM-H₂O₂ for 10 min, heated at 55 °C for 10 min (Murray & Williams, 1974) or treated with 10% (v/v) acetone (Sala-Trepat & Evans, 1971) confirmed that catechol was subject only to *ortho* cleavage.

Strain	Addition	Enzyme specific activities [nmol min ⁻¹ (mg protein) ⁻¹]					
		L(+)-Mandelate dehydrogenase	D(-)-Mandelate dehydrogenase	Phenylglyoxylate decarboxylase	Benzaldehyde dehydrogenase I	Benzyl alcohol dehydrogenase	Benzaldehyde dehydrogenase II
Wild-type EBF 65/65	None*	NA	ND	ND	ND	11	ND
	D(-)-Mandelate	NA	143	243	14	32	ND
	L(+)-Mandelate*	ND	ND	ND	ND	11	5
	Phenylglyoxylate	ND	137	493	39	18	ND
	Benzyl alcohol	ND	ND	ND	ND	212	43
C4201	Benzaldehyde	NA	ND	ND	ND	239	62
	Phenylglyoxylate	ND	210	531	79	NA	ND
	Benzyl alcohol	ND	ND	ND	ND	850	237
	Benzaldehyde	ND	ND	ND	ND	381	67
	Phenylglyoxylate	ND	ND	ND	ND	704	216
C4223	Benzaldehyde	ND	ND	ND	ND	459	116
	D(-)-Mandelate	ND	10	40	9	22	7
	Phenylglyoxylate	ND	65	455	41	9	ND
	Benzaldehyde	ND	ND	ND	ND	406	119
	D(-)-Mandelate*	ND	ND	7	ND	ND	ND
C4295	L(+)-Mandelate	67	43	293	50	139	30
	Phenylglyoxylate	91	97	490	67	13	ND
	Benzaldehyde	ND	ND	ND	ND	413	116
	D(-)-Mandelate*	ND	ND	445	30	21	ND
	Phenylglyoxylate	ND	ND	ND	ND	7	ND
C4413	Benzaldehyde	ND	ND	ND	ND	313	97

ND, not detectable [less than about 2 nmol min⁻¹ (mg protein)⁻¹]; NA, not assayed.

Table 4. *Reversion of auxotrophic and catabolic markers*

Reversion of auxotrophic markers was tested on minimal medium and reversion of catabolic markers on salts medium containing appropriate supplements.

Auxotrophic marker	Frequency of reversion	Catabolic marker	Frequency of reversion
<i>cys-3</i>	3.0×10^{-10}	<i>mdd-1</i>	2.4×10^{-9}
<i>ile-1</i>	5.5×10^{-9}	<i>mdd-2</i>	1.5×10^{-8}
<i>met-1</i>	$< 5.5 \times 10^{-9}$	<i>mdd-3</i>	$< 3.8 \times 10^{-10}$
<i>phe-1</i>	1.8×10^{-8}	<i>pgc-1</i>	4.8×10^{-9}
<i>thi-2</i>	1.8×10^{-8}		

of phenylglyoxylate (Table 3); it is possible that the mutation altered the uptake of D(-)-mandelate into the bacteria or in some way interfered with the operation of D(-)-mandelate dehydrogenase *in vivo*.

Mutant C4295 (*mdd-2 mdl-2*). Strains possessing a novel L(+)-mandelate dehydrogenase were isolated from a range of parental strains, including mutants in the mandelate pathway, at a frequency of about 2 to 4×10^{-10} (Vakeria, 1982). Mutant C4295 (Tables 2 and 3) was isolated as a spontaneous Mdl⁺ variant from the recombinant strain C4289 in order to construct a Mdd⁺Mdl⁺ strain suitable for examining possible linkage between the genes for the two mandelate dehydrogenases (although of course *mdl* mutations are not necessarily in the structural gene for L(+)-mandelate dehydrogenase). Mutant C4295, like C4226, could not grow on D(-)-mandelate (Table 2) although some D(-)-mandelate dehydrogenase activity was induced during growth in the presence of L(+)-mandelate or phenylglyoxylate (Table 3).

Mapping of catabolic markers

Mapping data were analysed in terms of absolute recombinant frequencies and of co-inheritance frequencies. Recombination frequencies were in the range 10^{-7} to 10^{-2} , well in excess of the frequencies of reversion (Table 4). Catabolic markers were tested as unselected donor markers (Table 5).

Overall, the map positions of auxotrophic markers agreed with those reported by Towner (1978) on the basis of his experiments with RP4. The chief differences were that *ala-1* and *cys-3* appeared to be in the opposite orientation to that found by Towner (1978) and *ile-1*, *met-1*, *ala-1*, *leu-2* and *cys-3* appeared to act as a linkage group, perhaps reflecting a feature of mobilization by pAV1 (Vakeria, 1982).

Examination of recombinants from some crosses showed that there was a considerable proportion lacking pAV1. This situation is analogous to that found for RP4 in *A. calcoaceticus* (Towner & Vivian, 1976b) and for R68.45 in *Rhizobium meliloti* (Kondorosi *et al.*, 1977) and *P. aeruginosa* (Haas & Holloway, 1978).

Mutant C4223 was used in the initial crosses and some of the recombinants obtained were used in subsequent mapping. Since in some recombinants, there was segregation of the ability to utilize D(-)-mandelate and phenylglyoxylate, two mutant alleles were designated in C4223: *mdd-1* and *pgc-1*. This mutant also lacked benzaldehyde dehydrogenase I activity (Table 3) but benzaldehyde was not included amongst the test substrates because poor and irreproducible growth on this rather inhibitory substrate made it difficult to characterize recombinants accurately. Mutant C4413 was also used in the early crosses in order to generate *mdd-3* recombinants containing auxotrophic markers. Three donor strains harbouring pAV1 were isolated in this way (C4435, C4437 and C4438) and these were in turn used in further crosses to determine co-inheritance of *mdd-3* with the various auxotrophic markers.

The position of *mdl-1* had been located by Towner (1978) between *phe-1* and *trp-2* using RP4. This location was confirmed in the present work from crosses involving pAV1. When *phe-1* was used as the selected donor marker, *pgc-1* showed 100% co-inheritance whereas *mdd-1*, *mdd-3*, *mdl-1* and *mdl-2* showed 70 to 79% co-inheritance. In at least two different crosses (DV57 and

Table 5. *Co-inheritance frequencies in crosses involving various mutant strains of A. calcoaceticus*

Recombinants were selected on appropriately supplemented minimal medium. Unselected catabolic markers were tested on appropriate salts medium.

Selected donor marker	Co-inheritance (%) of unselected donor marker with selected donor marker*		No. of colonies tested	Designation of cross
	<i>mdd-1</i>	<i>pgc-1</i>		
<i>cys-3</i> ⁺	27 (21-33)	47 (32-63)	194	DV55 and DV57: both C4408 × C4183
<i>met-1</i> ⁺	27 (20-33)	30 (27-33)	101	DV45: C4279 × C4290
<i>phe-1</i> ⁺	79 (64-94)	100	935	DV15 and DV22: both C4151 × C4223. DV31: C4185 × C4223. DV45: C4279 × C4290. DV55 and DV57: both C4408 × C4183. DV58: C4408 × C4290
<i>thi-2</i> ⁺	21 (15-26)	21 (15-26)	160	DV22: C4151 × C4223
	<i>mdd-3</i>			
<i>ile-1</i> ⁺	67 (58-75)		111	DV242: C4438 × C4664
<i>met-1</i> ⁺	68 (64-71)		106	DV242: C4438 × C4664
<i>phe-1</i> ⁺	79 (69-91)		325	DV224: C4435 × C484. DV225: C4437 × C484. DV226: C4438 × C484
<i>thi-2</i> ⁺	3 (0-10)		365	as for <i>phe-1</i> ⁺ selection
	<i>mdc-1</i>			
<i>ile-1</i> ⁺	76 (75-77)		159	DV228: C4440 × C4664. DV242: C4438 × C4664
<i>met-1</i> ⁺	76 (66-90)		154	as for <i>ile-1</i> ⁺ selection
<i>phe-1</i> ⁺	66 (65-67)		41	DV227: C4441 × C484
<i>thi-2</i> ⁺	0		111	DV227: C4441 × C484
	<i>mdl-1</i>			
<i>ile-1</i> ⁺	37		49	DV237: C4440 × C4248
<i>met-1</i> ⁺	38 (33-43)		99	DV237: C4440 × C4248
<i>phe-1</i> ⁺	70 (67-72)		84	DV229: C4439 × C484
<i>thi-2</i> ⁺	4 (3-4)		94	DV229: C4439 × C484
	<i>mdl-2</i>			
<i>cys-3</i> ⁺	36 (31-45)		194	DV55 and DV57: both C4408 × C4183
<i>phe-1</i> ⁺	77 (64-92)		420	DV55 and DV57 as above. DV58: C4408 × C4290
	<i>met-1</i>			
<i>ile-1</i> ⁺	94 (86-100)		1492	DV1a: C4151 × C4207. DV5: C4151 × C4201. DV10 and DV14: both C4151 × C4226. DV16: C4151 × C4664. DV17: C4151 × C48. DV48: C4294 × C4226. DV49: C4294 × C4201. DV61: C4151 × C4205. DV62: C4151 × C4420
	<i>ile-1</i>			
<i>met-1</i> ⁺	99 (95-100)		1091	DV1a, DV5, DV10, DV14, DV16, DV48, DV49, DV61, DV62 as above
	<i>cys-3</i>			
<i>met-1</i> ⁺	87 (77-96)		108	DV44: C4279 × C4289. DV45: C4279 × C4290
	<i>phe-1</i>			
<i>met-1</i> ⁺	29 (24-35)		153	DV44 and DV45 as above
	<i>met-1</i>			
<i>phe-1</i> ⁺	40 (34-46)		112	DV38: C4267 × C4103. DV44 as above
	<i>thi-2</i>			
<i>phe-1</i> ⁺	50 (44-55)		364	DV22: C4151 × C4223. DV31: C4185 × C4223

* Values in parentheses indicate the range of co-inheritance frequencies obtained from different crosses and counterselections.

DV58), a strain carrying *mdd-1* and *pgc-1* alleles was mated with a strain carrying *mdl-2*. Out of 420 colonies tested from the *phe-1*⁺ selection, 100% were Pgc⁺; 79% were Mdd⁺ and 94% of these Mdd⁺ colonies were also Mdl⁺. Similarly, on *cys-3*⁺ selection, 194 colonies were tested, of which about 27% were Mdd⁺, and 92% of these Mdd⁺ colonies were also Mdl⁺. It is not known why there was segregation between the *mdd-1* and *pgc-1* alleles when C4223 (lacking regulon-1 enzymes) was used in crosses. The results indicate that not only are *pgc-1* and *mdd-1* probably

Table 6. Analysis of the recombinants from a cross (DV242) involving strain C4438 (*ala-1 his-1 trp-2 mdd-3, pAV1*) and strain C4664 (*ile-1 met-1 mdc-1*)

Selection	No. of colonies tested	No. of inducible colonies	No. of colonies that were inducible and failed to grow on D(-)-mandelate
<i>ile-1</i> ⁺ / <i>his-1</i> ⁺	56	42	42
<i>ile-1</i> ⁺ / <i>trp-2</i> ⁺	55	41	32
<i>met-1</i> ⁺ / <i>his-1</i> ⁺	51	36	36
<i>met-1</i> ⁺ / <i>trp-2</i> ⁺	55	36	35
Total	217	155	145

linked but the *mdl-2* and *mdd-1* loci are probably also close together. This was the first indication of linkage between genes involved in the activities of D(-)-mandelate dehydrogenase and L(+)-mandelate dehydrogenase and is consistent with the co-ordinate physiological regulation of the two enzymes (Hills & Fewson, 1983*b*). The loci *mdl-1* and *mdl-2* may well be in the same gene.

Although the *mdd-2* mutation was used in eight different crosses its location was not clear since it failed to map consistently in one position.

Results from other crosses involving *mdd-3* and *mdc-1* alleles indicated that they did not appear to be located very close to *mdd-1*, *pgc-1*, *mdl-1* and *mdl-2*; *mdc-1* showed a slightly higher percentage co-inheritance with *ile-1* and *met-1* than with *phe-1*. Similarly, although *mdd-3* showed 79% co-inheritance with *phe-1*, it showed much higher co-inheritance with *ile-1* and *met-1* (67%) than did *mdd-1*, *pgc-1*, *mdl-1* or *mdl-2*. The markers *met-1*, *ile-1* and *cys-3* appear to be closely linked (Table 5). It is possible that *mdc-1* and *mdd-3* are located between *met-1* and *phe-1* with *mdc-1* closer to *met-1* and *mdd-3* closer to *phe-1*. A cross was made in which the donor strain carried the *mdd-3* mutation and was inducible (*mdc*⁺) for the regulon-1 enzymes. The recipient strain was mandelate-proficient (*mdd-3*⁺) but carried the *mdc-1* allele. Out of 217 recombinants analysed, 155 were found to be inducible and 94% of these inducible colonies had concomitantly gained the Mdd^{ns} phenotype (Table 6). This suggested possible linkage between *mdd-3* and *mdc-1* and it follows that the mutations *mdd-1* and *mdd-3* are probably located in different genes. This is further suggested by the isolation of Mdd^o recombinants at a frequency of 5×10^{-7} from a cross involving the *mdd-3* and *mdd-1/pgc-1* alleles; these colonies could not simply represent revertants because the reversion frequencies were low (Table 4).

Conclusions

The results from the mapping experiments described in the present paper suggest that, as with *P. putida* (Wheelis & Stanier, 1970; Wheelis, 1975) and *P. aeruginosa* (Rosenberg & Hegeman, 1969), several of the genes concerned with mandelate metabolism in *A. calcoaceticus* EBF 65/65 are chromosomal. Strain EBF 65/65 contains at least seven plasmids (Hinchliffe *et al.*, 1980; A. Vivian and D. Vakeria, unpublished results); however, 'curing' agents such as acriflavine, novobiocin or ethidium bromide failed to affect mandelate metabolism (Vakeria, 1982). Attempts to cure putative plasmids for mandelate or benzyl alcohol degradation in wild-type strain NCIB 8250 (which contains at least two plasmids; Vakeria, 1982) also failed (Fewson, 1978). Similarly, Keil *et al.* (1983) concluded that it was unlikely that there was a correlation between the ability of various strains of *Acinetobacter* to degrade certain aromatic carboxylic acids and the presence of plasmid DNA.

The mutants we have isolated are useful for physiological and biochemical studies but our mapping work has been hampered by a shortage of suitable mutants blocked in the catabolic pathways. Mapping studies with *P. putida* and *P. aeruginosa* also appear to have used rather few mandelate markers. We have experienced considerable difficulties in isolating appropriate mutants blocked in either the mandelate or the benzyl alcohol pathways from wild-type strains EBF 65/65 or NCIB 8250 but have had no problems in isolating auxotrophic mutants; it is not clear whether the difficulty has arisen as a result of failure to design suitable enrichment

techniques or as a consequence of some inherent property of the genetic regulation of the pathways. The present experiments have strengthened the impression gained from previous work that there is some sort of interaction between regulons 1 and 2.

The positions of the mandelate markers we have mapped so far in *A. calcoaceticus* indicate that there is unlikely to be a single operon; for instance *phe-1* intervenes between *mdc-1*, *mdd-3* and *pgc-1*, *mdd-1*, *mdl-1*, *mdl-2*. Nevertheless there is a considerable degree of gene clustering, as with the mandelate pathway of *P. aeruginosa* (Rosenberg & Hegeman, 1969). The apparent linkage between *mdd-1* and *mdl-1*, *mdl-2* strengthens the notion that the 'novel' mandelate dehydrogenases in *A. calcoaceticus* (in this case L(+)-mandelate dehydrogenase) arise as a result of expression of silent genes closely related to those coding for the 'original' mandelate dehydrogenases (Hills & Fewson, 1983*a*, *b*).

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REFERENCES

- AHLQUIST, E. F., FEWSON, C. A. & RITCHIE, D. A. (1975). The induction of mutants of *Acinetobacter calcoaceticus* NCIB 8250 and their selection by vancomycin. *Journal of General Microbiology* **91**, 338-344.
- BARTH, P. T. & GRINTER, N. J. (1977). Map of RP4 derived by insertion of transposon C. *Journal of Molecular Biology* **113**, 455-474.
- BEGGS, J. D. & FEWSON, C. A. (1977). Regulation of synthesis of benzyl alcohol dehydrogenase in *Acinetobacter calcoaceticus* NCIB 8250. *Journal of General Microbiology* **103**, 127-140.
- BHAT, S. G. & VAIDYANATHAN, C. S. (1976). Involvement of 4-hydroxymandelic acid in the degradation of mandelic acid by *Pseudomonas convexa*. *Journal of Bacteriology* **127**, 1108-1118.
- CHAKRABARTY, A. M. & GUNSALUS, I. C. (1969). Defective phage and chromosome mobilization in *Pseudomonas putida*. *Proceedings of the National Academy of Sciences of the United States of America* **64**, 1217-1223.
- CHAKRABARTY, A. M., GUNSALUS, C. F. & GUNSALUS, I. C. (1968). Transduction and the clustering of genes in fluorescent pseudomonads. *Proceedings of the National Academy of Sciences of the United States of America* **60**, 168-175.
- DEMEREC, M., ADELBERG, E. A., CLARK, A. J. & HARTMAN, P. E. (1966). A proposal for a uniform nomenclature in bacterial genetics. *Genetics* **54**, 61-76.
- ERRINGTON, J. (1981). *The genetics of Pseudomonas morsprunorum*. PhD thesis, Council for National Academic Awards, Thames Polytechnic, London.
- FEWSON, C. A. (1978). Repression of benzyl alcohol dehydrogenase after growth of *Acinetobacter calcoaceticus* on phenylglyoxylate is not caused by loss of a plasmid. *Proceedings of the Society for General Microbiology* **5**, 109.
- FEWSON, C. A., LIVINGSTONE, A. & MOYES, H. M. (1978). Mutants of *Acinetobacter calcoaceticus* NCIB 8250 constitutive for the mandelate enzymes. *Journal of General Microbiology* **106**, 233-239.
- HAAS, D. & HOLLOWAY, B. W. (1978). Chromosome mobilization by the R plasmid R68.45: a tool in *Pseudomonas* genetics. *Molecular and General Genetics* **158**, 229-237.
- HEGEMAN, G. D. (1966). Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. I. Synthesis of enzymes by the wild type. *Journal of Bacteriology* **91**, 1140-1154.
- HEGEMAN, G. D. & ROOT, R. T. (1976). The effect of a non-metabolizable analog on mandelate catabolism in *Pseudomonas putida*. *Archives of Microbiology* **110**, 19-25.
- HILLS, C. A. & FEWSON, C. A. (1983*a*). Mutant strains of *Acinetobacter calcoaceticus* possessing additional mandelate dehydrogenases. Identification and preliminary characterization of the enzymes. *Biochemical Journal* **209**, 379-386.
- HILLS, C. A. & FEWSON, C. A. (1983*b*). Regulation of expression of novel mandelate dehydrogenases in mutants of *Acinetobacter calcoaceticus*. *Journal of General Microbiology* **129**, 2009-2015.
- HINCHLIFFE, E. & VIVIAN, A. (1980*a*). Naturally occurring plasmids in *Acinetobacter calcoaceticus*: a P class R factor of restricted host range. *Journal of General Microbiology* **116**, 75-80.
- HINCHLIFFE, E. & VIVIAN, A. (1980*b*). Gene transfer in *Acinetobacter calcoaceticus*: fertility variants of the sex factor pAV1. *Journal of General Microbiology* **119**, 117-122.
- HINCHLIFFE, E., NUGENT, M. E. & VIVIAN, A. (1980). Naturally occurring plasmids in *Acinetobacter calcoaceticus*: pAV2, a plasmid which influences the fertility of the sex factor pAV1. *Journal of General Microbiology* **121**, 411-418.
- KEIL, H., TITTMANN, U. & LINGENS, F. (1983). Degradation of aromatic carboxylic acids by *Acinetobacter*. *Systematic and Applied Microbiology* **4**, 313-325.
- KEMP, M. B. & HEGEMAN, G. D. (1968). Genetic control of the β -ketoadipate pathway in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **96**, 1488-1499.
- KENYON, G. L. & HEGEMAN, G. D. (1979). Mandelate racemase. *Advances in Enzymology and Related Areas of Molecular Biology* **50**, 325-360.
- KONDOROSI, A., KISS, G. B., FORRAI, T., VINCZE, E. & BANFALVI, Z. (1977). Circular linkage map of *Rhizobium meliloti* chromosome. *Nature, London* **268**, 525-527.
- LEIDIGH, B. J. & WHEELIS, M. L. (1973). The clustering

- on the *Pseudomonas putida* chromosome of genes specifying dissimilatory functions. *Journal of Molecular Evolution* **2**, 235-242.
- LIVINGSTONE, A. & FEWSON, C. A. (1972). Regulation of the enzymes converting L-mandelate into benzoate in bacterium NCIB 8250. *Biochemical Journal* **130**, 937-946.
- LIVINGSTONE, A., FEWSON, C. A., KENNEDY, S. I. T. & ZATMAN, L. J. (1972). Two benzaldehyde dehydrogenases in bacterium NCIB 8250. Distinguishing properties and regulation. *Biochemical Journal* **130**, 927-935.
- MURRAY, K. & WILLIAMS, P. A. (1974). Role of catechol and the methylcatechols as inducers of aromatic metabolism in *Pseudomonas putida*. *Journal of Bacteriology* **117**, 1153-1157.
- NOVICK, R. P., CLOWES, R. C., COHEN, S. N., CURTISS, R., DATTA, N. & FALKOW, S. (1976). Uniform nomenclature for bacterial plasmids: a proposal. *Bacteriological Reviews* **40**, 168-189.
- ROSENBERG, S. L. & HEGEMAN, G. D. (1969). Clustering of functionally related genes in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **99**, 353-355.
- SALA-TREPAT, J. M. & EVANS, W. C. (1971). The meta cleavage of catechol by *Azotobacter* species. 4-Oxalocrotonate pathway. *European Journal of Biochemistry* **20**, 400-413.
- TOWNER, K. J. (1978). Chromosome mapping in *Acinetobacter calcoaceticus*. *Journal of General Microbiology* **104**, 175-180.
- TOWNER, K. J. & VIVIAN, A. (1976a). RP4-mediated conjugation in *Acinetobacter calcoaceticus*. *Journal of General Microbiology* **93**, 355-360.
- TOWNER, K. J. & VIVIAN, A. (1976b). RP4 fertility variants in *Acinetobacter calcoaceticus*. *Genetical Research* **28**, 301-306.
- VAKERIA, D. (1982). *Genetic aspects of mandelate catabolism in Acinetobacter calcoaceticus*. PhD thesis, Council for National Academic Awards, Thames Polytechnic, London.
- WHEELIS, M. L. (1975). The genetics of dissimilarity pathways in *Pseudomonas*. *Annual Review of Microbiology* **29**, 505-524.
- WHEELIS, M. L. & STANIER, R. Y. (1970). The genetic control of dissimilatory pathways in *Pseudomonas putida*. *Genetics* **66**, 245-266.