A Comparative Study of Acquired Amidase Activity in *Pseudomonas* Species

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(Received 2 December 1985; revised 19 February 1986)

Pseudomonas putida PP3 carrying dehalogenases I and II and *Pseudomonas aeruginosa* PAU3 carrying dehalogenase I coded for by plasmid pUU2 were able to grow on 2-monochloropropionic acid (2MCPA). Neither strain utilized 2-chloropropionamide (2CPA) as a carbon or nitrogen source for growth. Mutations in both strains to 2Cpa⁺ phenotypes (designated *P. putida* PPW3 and *P. aeruginosa* PAU5, respectively) involved the expression of an acquired 2CPA-amidase activity. The amidase followed by dehalogenase reactions in these strains constituted a novel metabolic pathway for growth on 2CPA. *P. putida* PPW3 synthesized a constitutive amidase of molecular mass 59 kDa consisting of two identical subunits of 29 kDa. For those amides tested this acquired enzyme was most active against chlorinated aliphatic amides, although substrate affinities (K_m) and maximum rates of activity (V_{max}) were poor. *P. aeruginosa* PAU5 acquired a 2Cpa⁺ phenotype by overproducing the A-amidase normally used by this species to hydrolyse aliphatic amides. The A-amidase had only slight activity towards 2CPA. However, with constitutive synthesis the mutant grew on the chlorinated substrates. Chloroacetamide (CAA) was a toxic substrate analogue for these *Pseudomonas* strains. A strain resistant to CAA was isolated from *P. aeruginosa* PAU5 when exposed to 1–10 mM-CAA. This mutant, *P. aeruginosa* PAU6, synthesized an inducible A-amidase. CAA-resistance depended upon the simultaneous expression of CAA-inducible amidase and dehalogenase activities.

INTRODUCTION

Our interest in acylamidase enzymes was a progression from the previous studies of the dehalogenase of *Pseudomonas putida* PP3. This organism was isolated following the spontaneous expression of two distinct dehalogenase enzymes which allowed the strain to grow on chlorinated aliphatic acids including 2,2-dichloropropionate (the herbicide Dalapon) and 2-monochloropropionate (2MCPA) (Senior *et al.*, 1976; Slater *et al.*, 1979). One of the dehalogenase genes (for fraction I dehalogenase) has been mobilized into both *Pseudomonas aeruginosa* PAC1 and *P. putida* PaW340 using the chromosome-mobilizing plasmid R68.44 (Weightman, 1981; Beeching *et al.*, 1983). Derivative strains containing the R' (dehalogenase I) plasmid pUU2 have been designated *P. aeruginosa* PAU3 and *P. putida* PPW2, respectively.

We have now investigated the chlorinated aliphatic amides, in particular 2-chloropropionamide (2CPA), as alternative growth substrates requiring both amidase and dehalogenase activity for their use as carbon and/or nitrogen sources. The strains used were the original dehalogenase-containing isolate *P. putida* PP3 and the dehalogenase I-containing transconjugant *P. aeruginosa* PAU3. The object of the study was to determine the nature of any amidase activity in these strains enabling them to grow on 2CPA.

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Abbreviations: CAA, chloroacetamide; 2CPA, 2-chloropropionamide; 2MCPA, 2-monochloropropionic acid.

In *P. aeruginosa* PAC1 the range of substrates hydrolysed and the expression of the rather non-specific A-amidase have been modified through selection of a series of mutants on defined media and by recombination of mutant genomes (Brown & Clarke, 1972; Smyth & Clarke, 1975; Turberville & Clarke, 1981). In the latter experimental work, evolution of *P. aeruginosa* PAC1 to grow on a novel substrate (butyramide) was brought full circle in that both the amidase enzyme and regulatory genes were altered to allow the new enzyme to be induced by the novel substrate.

A precedent for utilization of halogenated aliphatic amides as substrates was found by Kelly (1965) in a slow-growing, unidentified bacterium which could use fluoroacetamide and fluoroacetate as carbon sources. Ammonia and fluoride ions were released during growth on a fluoroacetamide, indicating the action of both amidase and dehalogenase enzymes. However, with *P. aeruginosa*, lacking dehalogenase activity but with an A-amidase, fluoroacetamide is an acetamide analogue yielding toxic fluoroacetate as a metabolite. This property was used in a positive selection procedure to isolate amidase-negative mutants (Clarke & Tata, 1973).

These investigations prompted work with our dehalogenase-carrying strains to test the toxicity of halogenated aliphatic amides and the mechanisms of resistance, if any. We have already demonstrated that mutants of *P. putida* PP3 arise at high frequencies in response to toxic levels of chloroacetic acids and that these mutants have altered permease and dehalogenase enzyme profiles (Slater *et al.*, 1985). By investigating the chlorinated aliphatic amides as carbon and nitrogen sources it was hoped that the model of amidase enzyme evolution described for *P. aeruginosa* PAC1 (Clarke, 1982) could be extended to other organisms and other substrates.

METHODS

Bacteria and growth conditions. *P. putida* PP3 has been described previously (Slater *et al.*, 1979). *P. aeruginosa* PAC1 was kindly provided by Professor P. H. Clarke (University College, London, UK) and was used as a recipient of the chromosome-mobilizing plasmid R68.44 from *P. putida* PP3 (Weightman, 1981; Beeching *et al.*, 1983). The R' derivative carried a fraction I dehalogenase gene as well as tetracycline (Tc), ampicillin (Ap) and kanamycin (Km) resistance markers. A transconjugant, designated *P. aeruginosa* PAU3, containing plasmid pUU2 which encoded dehalogenase I, had the phenotype $\text{AmiE}^+ \text{AmiR}^+ 2\text{Mcpa}^+ \text{Tc}^R \text{Ap}^R \text{Km}^R$. Mutants of *P. putida* PP3, designated PPW3, and *P. aeruginosa* PAU3, designated PAU5 and PAU6, are described in this paper.

The basic growth medium described previously (Slater *et al.*, 1979) was supplemented with either succinate (10 mM) or 2MCPA (14 mM) as carbon sources and NH_4^+ (8 mM) as the nitrogen source. Alternatively, media with either propionamide (14 mM) or 2CPA (14 mM) as combined carbon and nitrogen sources were used; in these cases NH_4^+ was omitted from the basic growth medium. Where necessary, antibiotics were filter sterilized and added to give final concentrations of $50 \mu\text{g ml}^{-1}$ (Km, Ap) and $100 \mu\text{g ml}^{-1}$ (Tc). Growth was at 30°C either in 100 ml volumes with shaking at 160 r.p.m. or in 6 litre volumes with magnetic stirring and aeration at approximately 100 ml min^{-1} . Strain identity was checked on King's A and B media (King & Phillips, 1978) and by identification of protein electrophoresis patterns. The frequencies of mutations on selective, defined media were expressed as fractions of total nutrient agar counts.

Dehalogenase and amidase enzyme activities. Whole-cell activities were determined in exponential or maximum population phase organisms washed and resuspended in 0.1 M-potassium phosphate buffer pH 8.0 to an OD_{600} of 0.6. Cell-free extracts were prepared by treating cells suspended in 0.1 M-phosphate buffer pH 7.4 with lysozyme [$1 \text{ mg (g wet cell wt)}^{-1}$] in 5 mM-EDTA. After 30 min at 30°C the pH was readjusted to 7.4, and 10 mM- MgSO_4 was added with 0.1 mg of type III deoxyribonuclease and type IA ribonuclease per g wet cells for an additional 30 min. The cell-free extract was centrifuged at $15000 g$ for 30 min at 4°C and the cleared supernatant used for amidase and dehalogenase assays. The amidase activity was measured as the rate of NH_3 release determined by the Berthelot reaction (Weatherburn, 1967; Friedrich & Mitrenga, 1981). The dehalogenase activity was determined by automatic potentiometric titration of free chloride ions (Slater *et al.*, 1979). The acyltransferase activity of the amidase fractions, with hydroxylamine as acyl-acceptor, was determined by an established method (Brammar & Clarke, 1964). Acetanilide hydrolysis was determined as the rate of aniline formation (Sharabi & Bordeleau, 1969). The potential substrates examined included: 2MCPA (Aldrich); 2CPA, chloroacetamide (CAA), acetamide and lactamide (Sigma); and valeramide and phenylacetamide, which were generous gifts from Professor P. H. Clarke (University College, London). All the amides were recrystallized once from ethanol.

Several lines of evidence suggest that low molecular mass amides diffuse across cell membranes (Clarke, 1980). Thus, initial amidase substrate activity profiles were determined in whole-cell assays using approximately mid-exponential phase organisms and substrate concentrations of 10 mM. Detailed enzyme kinetics were performed with partially purified amidase fractions and substrate concentrations in the range 0.1–400 mM. Enzyme constants were determined from linear regression analysis to Langmuir-Hanes plots ($([S])/v$ against $[S]$). Reaction rates of

denatured (autoclaved) controls were subtracted from observed rates. Protein concentrations were estimated by the Lowry method. The effects of enzyme inhibitors were determined after 10 min preincubation with either iodoacetamide (10 mM) (rate corrected for hydrolysis of the inhibitor), *N*-ethylmaleimide (10 mM), phenylmethylsulphonyl fluoride (2 mM), or *p*-hydroxymercuribenzoate (10 mM).

Enzyme separation and purification. Routine identification of amidase and dehalogenase enzymes was done by discontinuous polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions. Dehalogenase activity was located in gels by precipitation of hydrolysed chloride (Weightman & Slater, 1980). Acyltransferase activity was detected by incubating gels at 30 °C for 30 min with 100 mM-amide substrates, 250 mM-hydroxylamine in 0.2 M-Tris/HCl pH 7.9 followed by development in 0.4 M-FeCl₃ in 0.25 M-HCl. After activity staining the gels were stained for proteins overnight in 0.05% (w/v) PAGE blue G-90 (BDH) in 25% (v/v) acetic acid. The identity of acyltransferase and amidase activities was taken as tentative until these activities could be co-purified as indicated in the following procedures.

The A-amidase of *P. aeruginosa* PAU5 was purified as described by Brown *et al.* (1969) except that cell-free extracts, prepared by lysozyme lysis, were obtained from organisms grown in 3 litre batch culture with 2CPA as the carbon and nitrogen source.

The C-type amidase (as defined in Results) of *P. putida* PPW3 was purified from a 6 litre culture grown to maximum population phase on 14 mM-2CPA. Whole-cell amidase activity was monitored during the growth phases and the culture harvested at maximum expression. The soluble protein extract was fractionated at 0 °C with 40% and 60% saturation with (NH₄)₂SO₄. The 60% precipitate was dissolved in 0.1 M-phosphate buffer pH 7.4 and 1 mM-dithiothreitol (DTT). This fraction was desalted on a Sephadex G-15-120 (2 cm² × 30 cm) column and the eluate absorbed to a DEAE-Sephadex A50 (5.3 cm² × 30 cm) column equilibrated with 0.1 M-phosphate buffer and 1 mM-DTT. The column was eluted with 1 litre 0.1 M-phosphate buffer and 1.0 mM-DTT gradient ranging from 10 to 500 mM-KCl. The amidase-containing fractions were pooled and desalted as described above. This fraction was used for *V*_{max} and *K*_m determinations and inhibitor studies. For molecular mass determination a sample was concentrated by dialysis against 0.1 M-phosphate buffer pH 8.0 containing DTT and 10% (v/v) polyethylene glycol 6000 and eluted from a Sephadex G-150-120 (2 cm² × 90 cm) column precalibrated with blue dextran, bovine serum albumin, pepsin and lysozyme. The markers were also included in the amidase elution and *A*₂₈₀ and amidase activity were monitored.

Amidase molecular mass under SDS-denaturing conditions was estimated using a DEAE-Sephadex fraction purified to homogeneity by preparative PAGE on a Shandon column. The active fraction from this stage was applied to both linear (7.5%) and gradient (5–18%) SDS-PAGE gels with the same molecular mass markers as above, together with ovalbumin, trypsinogen and β-lactoglobulin.

CAA-resistant mutants. The frequency of CAA-resistant mutants of *P. aeruginosa* PAU5 was determined on solid medium containing succinate (10 mM), NH₄⁺ (5 mM) and CAA (10 mM). Isolated colonies of resistant mutants were cultured and amidase and dehalogenase activity in whole cells determined as described above.

RESULTS

Acquisition of the 2Cpa⁺ phenotype

P. putida PP3 and *P. aeruginosa* PAU3, which carried plasmid pUU2, both grew at similar rates on 2MCPA (Table 1). The *P. aeruginosa* strain also grew on propionamide, presumably by virtue of its A-type amidase, which Clarke (1980) showed was also responsible for growth on acetamide. In contrast *P. putida* PP3 did not grow on propionamide (Table 1) and it was also shown that it could not grow on acetamide, butyramide, phenylacetamide or acetanilide. Neither pseudomonad was able to utilize 2CPA as either a carbon or a nitrogen source.

Initial observations suggested that cell-free extracts of both *P. putida* PP3 and *P. aeruginosa* PAU3 did not contain a 2CPA-amidase activity. However, closer examination showed that significant, low activities could be detected toward 2CPA in *P. aeruginosa* PAU3 grown on propionamide (Table 1). Similarly, if succinate-grown cells of *P. putida* PP3 were exposed to 14 mM-2CPA in resting cell suspensions, a very low 2CPA-amidase activity was measured (Table 1). However, both activities were substantially below the levels required to support growth on 2CPA.

When both parent strains were separately spread on medium containing 2CPA as both the carbon and nitrogen source, mutants appeared at frequencies of 4.7×10^{-7} (for *P. putida* PP3) and 1.1×10^{-5} (for *P. aeruginosa* PAU3). Two representative mutants, *P. putida* PPW3 and *P. aeruginosa* PAU5, were examined in detail and found to have growth rates on 2MCPA similar to

Table 1. *Growth rates and cell-free extract amidase activities*

The specific growth rates were determined during exponential growth phase. Amidase activities were measured in maximum population phase cell-free extracts with 80 mM-2CPA, except *a*, which was measured in cell-free extracts of succinate-grown cells exposed to 14 mM-2CPA for 4 h (during this period no growth of *P. putida* PP3 occurred). Enzyme activities are means of at least three determinations (with variations of less than 10% from the mean in all cases).

Strain	Growth substrate	Specific growth rate, $\mu_{\max}$ (h ⁻¹)	2CPA-amidase specific activity (nmol min ⁻¹ mg ⁻¹)
<i>P. putida</i> PP3	Succinate	0.56	0.24
	Propionamide	0	ND
	2MCPA	0.32	0.03
	2CPA	0	0.27 ^a
<i>P. putida</i> PPW3	Succinate	0.56	19.0
	Propionamide	0	ND
	2MCPA	0.32	23.0
	2CPA	0.12	120.0
<i>P. aeruginosa</i> PAU3	Succinate	0.44	0
	Propionamide	0.32	1.7
	2MCPA	0.30	0
	2CPA	0	ND
<i>P. aeruginosa</i> PAU5	Succinate	0.44	31.0
	Propionamide	0.31	39.0
	2MCPA	0.28	140.0
	2CPA	0.22	250.0

ND, Not determined.

their parents (Table 1). Both strains were also able to grow on 2CPA, albeit at lower specific growth rates than on 2MCPA (Table 1).

Both mutants produced substantial 2CPA-amidase activity, with the maximum enzyme specific activities present in cells taken from late exponential growth phase. 2CPA-amidase activity was constitutive in both mutants since substantial activities were measured in organisms grown on succinate or 2MCPA (Table 1). However, the activity in *P. putida* PPW3 grown on 2CPA was approximately fivefold greater than in organisms grown on succinate or 2MCPA. Similarly the activity in 2CPA-grown *P. aeruginosa* PAU5 was eight times greater than that in succinate-grown organisms. Interestingly, growth of *P. aeruginosa* PAU5 on 2MCPA produced substantially higher levels of 2CPA-amidase, which were about 4.5-fold greater than in succinate-grown cells and over half the level seen in 2CPA-grown organisms (Table 1). These observations are discussed later in the context of acquired resistance to CAA.

The positions of 2CPA-acyltransferase and 2MCPA-dehalogenase activities in PAGE-resolved proteins are shown in Fig. 1. Duplicate gels were separately stained for activity of the two enzymes and their positions marked. Subsequently the same gels were stained for protein. As expected, both strains contained the fraction I dehalogenase, but the fraction II dehalogenase was only found in *P. putida* PPW3. The acyltransferase activities of *P. putida* PPW3 and *P. aeruginosa* PAU5 showed different electrophoretic mobilities (Fig. 1). The mobility of the PAU5 acyltransferase was identical to that observed for *P. aeruginosa* strains PAU3 and PAC1 grown on propionamide. This suggested that the 2CPA-acyltransferase activity was due to the normal A-amidase synthesized by *P. aeruginosa*. To confirm this, substrate profiles against a range of amide substrates for the amidases from the three *P. aeruginosa* strains were obtained. The activity ratios for *P. aeruginosa* strains PAC1, PAU3 and PAU5 were identical, the only difference being, as expected, that the specific activity of the enzyme in strain PAU5 was about 150 times greater than in the other two strains. Thus it is clear that the enhanced 2CPA-amidase activity of *P. aeruginosa* PAU5, enabling it to grow on 2CPA as a carbon, energy and nitrogen source, was due to elevated levels of A-amidase expression. Indeed it was apparent in PAGE gels (Fig. 1), and subsequently during the purification step,

Fig. 1

Fig. 2

Fig. 1. Linear (7.5%) discontinuous PAGE of cell-free extracts of *P. aeruginosa* PAU5 (lane 1, 170 µg protein; lane 2, 60 µg protein) and *P. putida* PPW3 (lane 3, 70 µg protein; lane 4, 200 µg protein) grown on 2CPA. Gels were first stained for acyltransferase activity (open arrows) with 100 mM-2CPA or for dehalogenase activity (filled arrows) with 100 mM-2MCPA. The top filled arrow indicates dehalogenase I activity in both strains. The bottom filled arrow shows the position of dehalogenase II activity present in *P. putida* PPW3 only. The gels were then stained for protein.

Fig. 2. Protein-stained gradient (5–18%) discontinuous SDS-PAGE of purified *P. putida* PPW3 C-amidase (lane 2) compared with standard proteins (lane 1) which are, from the bottom: lysozyme (14.3 kDa); β -lactoglobulin subunit (18.4 kDa); trypsinogen (24.0 kDa); pepsin (34.7 kDa); and bovine serum albumin (66.0 kDa).

that the A-amidase of strain PAU5 was the major soluble protein in 2CPA-grown organisms. These high levels of expression were necessary for growth since under substrate-saturated conditions the A-amidase showed a rate of hydrolysis of 2CPA which was only 2.5% the rate of hydrolysis of propionamide.

The acquired amidase of *P. putida* PPW3 not only showed a different electrophoretic mobility to the A-amidase but also exhibited an entirely different activity profile (Fig. 3). In contrast to the A-amidase, this enzyme showed a greater degree of specificity towards chlorinated aliphatic amides, with little activity towards unsubstituted amides and none against phenylacetamide or acetanilide. The enzyme was termed a C-amidase. 2CPA was the only amide which supported growth of strain PPW3 although a number of alkanolic acid derivatives including acetate, propionate, lactate and 2MCPA served as carbon and energy sources.

All the amide substrates were also tested for acyltransferase activity in cell-free extracts using hydroxylamine as an acyl acceptor. As already indicated in Fig. 1, acyltransferase activities

Fig. 3. Acyltransferase activities for different amide substrates (10 mM) in cell-free extracts of (a) *P. aeruginosa* PAC1 and (b) *P. putida* PPW3. Similar profiles to that in (a) were obtained for *P. aeruginosa* strains PAU3 and PAU5. The amide substrates were: PA, propionamide; IBA, isobutyramide; LA, lactamide; 2CPA, 2-chloropropionamide; AA, acetamide; CAA, chloroacetamide; BA, butyramide; VA, valeramide.

Table 2. Purification of the *P. putida* PPW3 C-amidase

Fraction	Volume (ml)	Protein (mg)	Total activity ($\mu\text{mol min}^{-1}$)	Yield (%)	Specific activity ($\text{nmol min}^{-1} \text{mg}^{-1}$)	Purification factor
Cell-free extract	195.0	620.0	14.0	100	22.6	1.0
Ammonium sulphate 40–60% saturation, followed by G-15 gel filtration	25.0	213.0	9.0	64	42.3	1.9
DEAE-Sephadex 50 fractions 22–25	100.0	30.6	8.4	60	276.0	12.0
Preparative PAGE	15.5	2.5	2.3	17	914.0	40.0

were detected in non-denaturing PAGE gels of *P. putida* PPW3 and *P. aeruginosa* PAU5 with 2CPA as the substrate. Strains PAU3 and PAU5 showed acyltransferase activities with aliphatic amides which were consistent with published data (Brown *et al.*, 1969; Brown & Clarke, 1972). The halogenated amides, 2CPA and 2CAA, were found to be unstable in the acyltransferase assay system, with high rates of non-enzymic reaction to form chloro-acylhydroxamate products. For this reason quantitative measurements of activity could not be made. However, both *P. aeruginosa* strains and *P. putida* PPW3 had slight acyltransferase activity with 2CPA and 2CAA as substrates.

Properties of the *P. putida* PPW3 amidase

The novel C-type amidase of *P. putida* PPW3 was purified to homogeneity as judged by linear and gradient gel electrophoresis (Table 2, Fig. 2). The final yield was 17% of the original activity after preparative PAGE, which gave an overall purification of 40-fold. The C-amidase of *P. putida* PPW3 had an apparent molecular mass of 59 kDa as determined by elution through a precalibrated Sephadex G-150 column. Resolution of the active C-amidase protein from the DEAE-Sephadex pooled fraction by preparative PAGE is shown in Fig. 4. Denaturing SDS-PAGE of fractions 16 and 17 from the preparative PAGE together with protein markers gave a subunit molecular mass of 29 kDa. An SDS-PAGE gradient (5–18%) gel of the purified C-amidase is shown in Fig. 2. Both amidase and acyltransferase activities were co-purified to the preparative PAGE step, indicating that they were due to one protein.

Fig. 4. Resolution of the C-amidase of *P. putida* PPW3 by preparative PAGE (7.5% gel) on a Shandon column. ○, Protein concentration; ■, C-amidase activity against 2CPA. No activity was detected in the two major protein peaks before fraction 10 or in the major peaks after fraction 20.

Using partially purified DEAE-Sephadex fractions of both the A- and C-amidases, the K_m values for major amide substrates were determined. Acyltransferase activity could not be accurately determined for the chlorinated amide substrates due to interference from the non-enzymic reaction. The C-amidase had its greatest affinity for 2CAA, with a K_m of 30 mM. The K_m for 2CPA was 206 mM. The A-amidase had K_m values for 2CPA and 2CAA of 80 mM and 25 mM, respectively. The C-amidase K_m for acetamide was calculated to be in the molar range, whereas the A-amidase K_m for acetamide was 100 μ M.

The V_{max} for the C-amidase for 2CPA was 1890 $\text{nmol min}^{-1} \text{mg}^{-1}$, which was at least 10 times greater than the V_{max} for any of the enzyme's other substrates. The V_{max} for the A-amidase for 2CPA was 78 $\text{nmol min}^{-1} \text{mg}^{-1}$, a rate which was one-tenth of the activity towards acetamide and nearly 20 times smaller than the V_{max} for 2CAA.

The effects of four enzyme inhibitors on the activity of the C-amidase hydrolysis of 2CPA were examined. *p*-Hydroxymercuribenzoate and *N*-ethylmaleimide at 10 mM significantly inhibited the C-amidase, indicating the possible involvement of thiol functional groups in stabilizing enzyme structure or at the active site. Iodoacetamide, a weak substrate for the enzyme, but also an alkylating agent, had no effect on the rate of hydrolysis of 2CPA.

Mutation of P. aeruginosa PAU5 to growth in the presence of CAA

For the strains able to grow on 2CPA, it was found that CAA was a toxic analogue of 2CPA. The toxicity was probably due to the intracellular release of chloroacetic acid following the amidase reaction since strains lacking the amidase enzyme were much less sensitive to inhibition. An exponentially growing culture of *P. aeruginosa* PAU5 was spread onto succinate minimal media containing CAA varying from 1 to 20 mM. Inhibition of growth occurred at all CAA concentrations. However, after at least 3 d incubation, CAA-resistant mutants of *P. aeruginosa* PAU5 arose at frequencies of 5.9×10^{-6} on media containing 1–10 mM-CAA. One mutant, designated *P. aeruginosa* PAU6, was resistant to up to 15 mM-CAA.

Strains PAU5 and PAU6 were grown to maximum population phase in succinate (10 mM) minimal medium containing limiting amounts of ammonia (0.7 mM) and the whole-cell 2MCPA-dehalogenase and 2CPA-amidase activities were determined (Table 3). The cultures were then split and exposed to CAA, 2CPA or 2MCPA and ammonia for 4 h after which the culture OD_{600} , 2MCPA-dehalogenase and 2CPA-amidase activities were determined. The results showed that the constitutive A-amidase activity of *P. aeruginosa* PAU5 was lost in strain PAU6 since no amidase activity could be detected in succinate-grown cells (Table 3). The A-amidase activity of strain PAU5 was enhanced ninefold by exposure to 2MCPA (see Table 1).

For both strains high dehalogenase levels were induced by 2MCPA, CAA and 2CPA. In strain PAU5 amidase activity in the non-growing cells exposed to CAA and 2CPA was less than

Table 3. *Whole-cell 2CPA-amidase and 2MCPA-dehalogenase activities for P. aeruginosa strains PAU5 and PAU6*

The enzyme activities were determined in growing or starving cultures after harvesting and resuspending the cells in 0.1 M-phosphate buffer to a standard OD₆₀₀ of 0.6.

Strain	Growth conditions	Enzyme activity (nmol min ⁻¹ ml ⁻¹)	
		Amidase	Dehalogenase
<i>P. aeruginosa</i> PAU5	Succinate + NH ₄ ⁺	4.9	0
	2MCPA + NH ₄ ⁺	44.7	46.7
	CAA	1.8	8.5
	2CPA	2.3	14.0
<i>P. aeruginosa</i> PAU6	Succinate + NH ₄ ⁺	0	0
	2MCPA + NH ₄ ⁺	1.4	71.2
	CAA	1.6	26.9
	2CPA	2.6	23.2

the constitutive levels seen in succinate-grown cells. However, for the mutant PAU6, CAA and 2CPA caused induction of amidase activity. Thus, the significant change in the mutation from CAA sensitivity to CAA resistance was a return to a regulated amidase phenotype from the constitutive amidase synthesis in *P. aeruginosa* PAU5. The CAA-resistant mutant *P. aeruginosa* PAU6 retained its 2Cpa⁺ growth phenotype.

DISCUSSION

Low levels of 2CPA-amidase activity could be detected in *P. putida* PP3 and *P. aeruginosa* PAU3 exposed to 2CPA or propionamide, respectively. Neither strain was able to utilize 2CPA as a carbon, energy or nitrogen source. Mutants were readily obtained which altered their activity towards 2CPA, enabling growth to occur on this substrate, probably as a result of mutations in the regulatory mechanisms.

In the case of *P. putida* PP3 mutation to a 2Cpa⁺ phenotype involved the constitutive synthesis of a novel C-amidase which was active toward a limited range of chlorinated aliphatic amides. The C-amidase was only slightly active toward propionamide and did not hydrolyse acetamide, phenylacetamide or acetanilide. The C-amidase of *P. putida* represents a novel amidase, with a distinct molecular mass and subunit structure compared with other amidases described for *Pseudomonas* species. The A-amidase of *P. putida* biotype A strains has been described as being similar to the enzyme found in *P. aeruginosa* (Clarke, 1972). The latter is a hexameric protein of subunits with molecular mass 40 kDa. The acetanilide-hydrolysing enzyme of *P. acidovorans* has an active molecular mass of 57 kDa, very similar to the C-amidase described here. However, it has a monomeric structure (Alt *et al.*, 1975). The molecular structures of other *Pseudomonas* amidases have not been determined.

The relatively high *K_m* for 2CPA of the C-amidase in *P. putida* PPW3 perhaps suggests that expression involved recruitment of an enzyme that had evolved for some other unknown function. This function may have involved hydrolysis of other amide or ester bonds, but since hydrolysis reactions are common it is unlikely that the normal physiological role of the C-amidase will be identified.

For *P. aeruginosa* PAU3 the nature of its amidase activity lay in the well-characterized A-amidase which hydrolyses the aliphatic amides formamide, acetamide or propionamide. The A-amidase was inducible in the parental strain, but to levels which were not high enough for growth on the poor substrate 2CPA. Mutation to the 2Cpa⁺ phenotype resulted in enhanced synthesis of the A-amidase to levels which were 150 times higher than in the propionamide-induced parental strain. This high level of expression was constitutive, although an eightfold increase in the specific activity present in succinate- or propionamide-grown cells was achieved by growing the mutant *P. aeruginosa* PAU5 on 2CPA. Some increased expression of the A-

amidase (4.5-fold) was also achieved by growing strain PAU5 on 2MCPA. The reason for the elevated levels of constitutive synthesis by a non-amide substrate remains obscure, but it may be due to different levels of catabolic repression depending on the growth substrate.

Growth of *P. aeruginosa* PAU5 required two sequential steps to remove ammonia and chloride. The pathway was constructed in strain PAU5 by first introducing a dehalogenase I enzyme coded for by plasmid pUU2 transferred from *P. putida* PP3. The 2CPA-amidase activity was then acquired by selection of an A-amidase regulatory mutant, through the selection of a constitutive mutant. It is interesting to note that the appearance of this mutant would in itself not have generated a novel growth phenotype in the absence of the initial dehalogenase I transfer. Furthermore, it is known that this strain of *P. aeruginosa* does not contain any dehalogenase activity whether originally expressible or cryptic. Acquisition of the capacity to use 2CPA as a growth substrate led concomitantly to sensitivity to toxic levels of some chlorinated aliphatic amides, in particular CAA. This is similar to the acquired sensitivity obtained when *P. putida* PP3 evolved the capacity to use halogenated alkanolic acids for growth (Slater *et al.*, 1985). It is likely that CAA toxicity in *P. aeruginosa* PAU5 is due to the intracellular production of monochloroacetic acid, which is known to inhibit growth of pseudomonads (Slater *et al.*, 1979, 1985). CAA-resistant mutants, such as *P. aeruginosa* PAU6, were readily isolated and could have been due to: mutations in the uptake system excluding CAA from the cell; or deletion of the A-amidase; or a change in the regulatory properties of the amidase and/or dehalogenase. In all cases these mechanisms would ensure that there was no inhibitory accumulation of toxic metabolites such as monochloroacetic acid. The resistant mutants, such as *P. aeruginosa* PAU6, were shown to have retained the 2Cpa⁺ phenotype and could not have acquired a CAA resistance phenotype as a result of deleting, or otherwise eliminating, A-amidase activity. Thus the mutants were unlike the fluoroacetamide mutants isolated by Clarke & Tata (1973) giving resistance to this toxic amide. Instead resistance to CAA appeared to be due to a return to a regulatory function to the A-amidase. Constitutive expression in the parent *P. aeruginosa* PAU5 was replaced by a chlorinated amide inducible amidase system (Table 3).

This paper demonstrates that a simple novel metabolic pathway may be constructed by several different mechanisms. Dehalogenase I transfer by a plasmid-mediated mechanism was necessary since *P. aeruginosa* does not contain a gene for the structural dehalogenase enzyme. The typical selection of a non-regulated constitutive mutant led to the formation of the simple two-step pathway. And finally interaction with other toxic analogues led to the restoration of a regulated pathway.

We gratefully acknowledge fellowship support for R. C. Wyndham from the Natural Science and Engineering Research Council of Canada.

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