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Catabolism of Aromatic Biogenic Amines by *Pseudomonas aeruginosa* PAO1 via *meta* Cleavage of Homoprotocatechuic Acid

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***Pseudomonas aeruginosa* PAO1 catabolized the aromatic amines tyramine and octopamine through 4-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid (HPA). *meta* ring cleavage was mediated by 3,4-dihydroxyphenylacetate 2,3-dioxygenase (HPADO), producing 2-hydroxy-5-carboxymethylmuconic semialdehyde (MSA). An NAD-dependent dehydrogenase caused the disappearance of the yellow MSA product, probably forming 2-hydroxy-5-carboxymethylmuconic acid. Induction studies with extracts from mutant cells indicated that the inducer of HPADO was HPA and/or MSA. Strains PAO1.221 (*tynC1*) and PAO1.303 (*tynD1*) have chromosomal mutations causing a deficiency in the activity necessary for conversion of 4-hydroxyphenylacetic acid to HPA. Genetic analyses showed that the mutations were in different loci. Strains PAO1.197 (*tynE1*) and PAO1.185 (*tynF1*) are deficient in HPADO and the NAD-dependent dehydrogenase, respectively. Plasmid pRO1853 was constructed by cloning approximately 7.3 kilobases of PAO1 chromosomal DNA into the *Bam*HI site of the vector plasmid pRO1614. This recombinant plasmid complemented the *tynD1*, *tynE1*, and *tynF1* mtations. A putative repressor-binding site involved in the regulation of HPADO synthesis was observed for a subcloned fragment of pRO1853. This recombinant plasmid, pRO1863, failed to complement *tynE1* or *tynF1* but still complemented *tynD1*. Another construct, pRO1887, contained 9.2 kilobases of PAO1 chromosomal DNA inserted in the *Pst*I site of the vector pRO1727. Plasmid pRO1887 complemented only the *tynC1* mutation. Mapping experiments performed with the chromosome-mobilizing plasmid R68.45 located the mutations described above in a cluster at about 35 to 40 min of the PAO1 chromosome map. The mutations were linked to the *proA*, *thr-48*, *lys-9015*, *argF10*, and *argG* markers.**

Aromatic biogenic amines such as tyramine (Fig. 1), dopamine, and norepinephrine are present in nature, where, for example, they are important components of the mammalian nervous system. Despite this, little is known of their catabolism, particularly by bacteria. These compounds can be divided into two categories, phenethylamines and phenethanolamines, based on whether the alkylamine side chain is hydroxylated. Previously, we have shown that the route of initial catabolism of these compounds by *Pseudomonas aeruginosa* PAO1 was category dependent (11). Phenethylamine catabolism was initiated by a membrane-bound tyramine dehydrogenase and, with tyramine as the substrate, and yielded 4-hydroxyphenylacetaldehyde (4HPAL) as the product. Transformation of tyramine dehydrogenase-negative mutants with cloned portions of the PAO1 chromosome distinguished two mutant classes. The respective mutations of each class were closely linked and mapped at about 75 to 80 min on the PAO1 chromosomal map. Phenethanolamine catabolism was initiated by a soluble, oxygen-independent, pyridine nucleotide-independent enzyme tentatively described as octopamine hydrolyase. The product of the reaction, with octopamine as the substrate, was also 4HPAL (Fig. 1). A soluble, NAD-dependent 4HPAL dehydrogenase converted 4HPAL to 4-hydroxyphenylacetic acid (4HPAA). No mutants devoid of these latter two enzymes were isolated.

Bacterial catabolism of 4HPAA proceeds via ring hydroxylation forming either 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid [HPA]) (8, 35) or 2,5-dihydroxyphenylacetic acid (homogentisic acid [HGA]) (4, 16). Another route through a trihydroxylated intermediate, 2,4,5-trihydroxyphenylacetic acid, has been reported in the yeast *Trichosporom cutaneum* (34), whereas catabolism of phenylalanine in *Aspergillus niger* proceeds through 4HPAA, 4-hydroxymandelic acid, and protocatechuate (20). Dioxygenases cleave the aromatic rings of these compounds, and further metabolism yields tricarboxylic acid cycle intermediates (7, 35).

We report in the present study that catabolism of biogenic amines by *P. aeruginosa* PAO1 proceeded from 4HPAA through HPA, which underwent *meta* cleavage between the second and third ring carbon atoms to form 2-hydroxy-5-carboxymethylmuconic semialdehyde (MSA). We isolated mutants deficient in each enzyme of this part of the pathway and show in this study that the mutations were clustered at about 35 to 40 min on the PAO1 chromosomal map. We also cloned portions of the PAO1 chromosome which complemented each of the mutations and, through subcloning experiments, localized a putative regulatory region.

(Preliminary accounts of this work have been presented [S. M. Cuskey and R. H. Olsen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K36, p. 177, and 1986, K190, p. 225].)

MATERIALS AND METHODS

Bacterial strains and growth media. Bacteria and plasmids used in this study are listed in Table 1. All mutant strains were derived from *P. aeruginosa* PAO1 (17) and were isolated after exposure to nitrosoguanidine by using standard techniques. All media and growth conditions were as previously described (11).

Extract preparation and enzyme assays. Cell extracts used in enzyme assays were prepared from cells cultured in 100 ml of the appropriate salts medium as previously described

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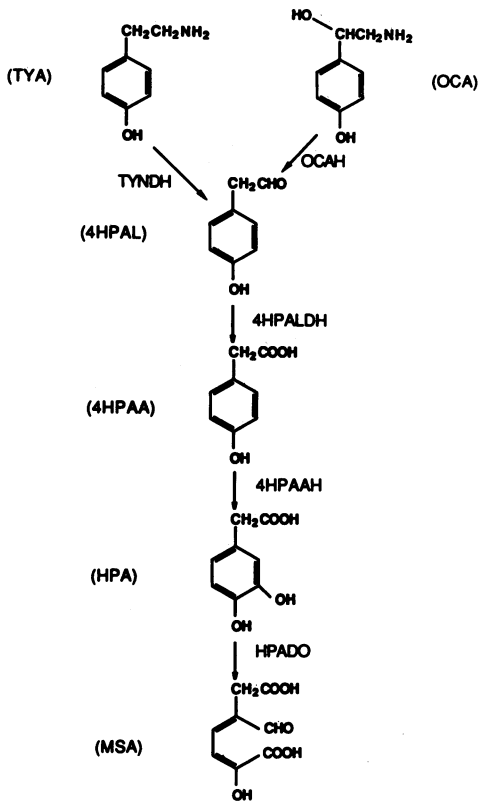

FIG. 1. Proposed pathway of aromatic amine catabolism in *P. aeruginosa* PAO1. Compounds: TYA, tyramine; OCA, octopamine. Enzymes: TYNDH, tyramine dehydrogenase; OCAH, octopamine hydrolyase; 4HPALDH, 4-hydroxyphenylacetaldehyde dehydrogenase; 4HPAAH, hydroxyphenylacetate 3-hydroxylase.

(11). Cells were disrupted by treatment with a probe-type ultrasonic oscillator (18). The soluble supernatant fraction of the ruptured cells was collected after centrifugation (105,000 × *g*, 2 h, 4°C), and the cell extract was used in all enzyme assays. Assays for homoprotocatechuic acid 2,3-dioxygenase (HPADO) were performed as described elsewhere (21), and results are expressed as nanomoles of product formed per minute per milligram of protein. 2-Hydroxy-5-carboxymethylmuconic semialdehyde dehydrogenase (MSADH) activity was determined by adding NAD (final concentration, 2 mM) to reaction mixtures in which MSA formation from HPA was maximal and by measuring loss of absorbance at 380 nm. In these cases, the concentration of substrate HPA was adjusted so that total conversion to the semialdehyde product occurred after several minutes of incubation. Because this resulted in a relatively low concentration of semialdehyde and because the semialdehyde is relatively unstable, MSADH activity is reported qualitatively in the text. Protein was measured by the method of Bradford (6).

Genetic techniques. Chromosomal mapping experiments involving mutants unable to utilize tyramine or 4HPAA as the sole source of carbon were performed with the chromosome-mobilizing plasmid R68.45 and variously marked mutant strains by using standard techniques (31).

Plasmids pRO1614 (30), pRO1727 (11), and pRO1769 (10) were used as vectors for the cloning and subcloning of PAO1 chromosomal DNA in these experiments. Techniques for DNA isolation and purification (30), restriction endonuclease cleavage and ligation of purified DNA (10), bacterial cell transformations (30), rapid cell lysis for visualization of recombinant plasmids (13), and agarose gel electrophoresis (10) have been described elsewhere.

Chemicals and reagents. Aromatic compounds used in growth studies, enzyme induction, and enzyme assays were of the highest purity commercially available from Sigma Chemical Co., St. Louis, Mo., or Aldrich Chemical Co., Inc., Milwaukee, Wis. Antibiotics were purchased from Sigma. Complex medium components and agar were purchased from Difco Laboratories, Detroit, Mich. Restriction endonucleases and other enzymes involved in DNA manipulations were purchased from International Biotechnologies Inc., New Haven, Conn., or Bethesda Research Laboratories, Gaithersburg, Md.

RESULTS

Mutant isolation and characterization. Recently (11), we described a pathway for the catabolism of the phenethylamine tyramine and the phenethanolamine octopamine that utilized 4HPAA as an intermediate (Fig. 1). Mutants unable to utilize tyramine and 4HPAA as the sole sources of carbon were isolated to investigate the route of further catabolism of these compounds (Table 2). Mutant strains PAO1.221 (*tynC1*) and PAO1.303 (*tynD1*) were unable to utilize 4HPAA but grew at wild-type rates on the possible pathway intermediates HPA and HGA. Both mutants also catabolized the 3,4-dihydroxylated aromatic amines, dopamine and norepinephrine. Cells of either mutant grown in the presence of tyramine accumulated 4HPAA in the culture medium (by thin-layer chromatography and high-pressure liquid chromatography; data not shown). This result indicated that strains PAO1.221 and PAO1.303 contain lesions in genes responsible for the catabolism of 4HPAA mediated by a putative 4HPAA hydroxylase. Genetic analysis demonstrated that the *tynC1* and *tynD1* mutations were in different loci (see below).

Mutant strains PAO1.197 (*tynE1*) and PAO1.185 (*tynF1*) failed to utilize 4HPAA, HPA, or any of the aromatic amines tested but grew at wild-type rates on HGA (Table 2). This indicated that the metabolism of the aromatic amines proceeds through HPA. When grown on lactate in the presence of 4HPAA (or tyramine), strain PAO1.197 accumulated HPA in the medium (by thin-layer chromatography and high-pressure liquid chromatography; data not shown), and when grown with the same compounds, strain PAO1.185 accumulated a yellow product resembling a muconic semialdehyde. These data suggest that mutant PAO1.197 is deficient in an enzyme mediating HPA *meta* ring cleavage and that mutant PAO1.185 is deficient in the enzyme mediating further catabolism of the ring cleavage product.

Enzymatic analysis. Previous reports (1, 2, 8, 24) indicated that 4HPAA conversion to HPA is mediated by a 4-HPAA 3-hydroxylase. However, extracts of wild-type cells induced by 4HPAA, prepared either by sonication or by passage through a French pressure cell, incubated with NAD(P)H and 4HPAA, with or without flavine adenine dinucleotide, flavine mononucleotide, or 6-methyl-5,6,7,8-tetrahydropterine (27), failed to show 4HPAA-dependent hydroxylase activity as measured spectrophotometrically by the disappearance of NAD(P)H or by oxygen consumption. Thin-layer chromatography analysis of reaction mixtures incubated overnight showed no disappearance of the substrate and no accumulation of HPA (data not shown).

Soluble extracts of wild-type, tyramine-induced cells formed a yellow product when they were incubated with

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description, genotype, or phenotype[a]	Derivation, source, or reference[b]
Strains		
PAO1	Prototroph	Laboratory stock; 17
PAO1.132	*vma-1*	11
PAO1.221	*vma-1 tynC1*	PAO1.132, NTG, Tya$^-$; this study
PAO1.303	*vma-1 tynD1*	PAO1.132, NTG, Tya$^-$; this study
PAO1.197	*vma-1 tynE1*	PAO1.132, NTG, Tya$^-$; this study
PAO1.185	*vma-1 tynF1*	PAO1.132, NTG, Tya$^-$; this study
PAO25	*argF leu-10*	17
PAO236	*hisII ilvBC lys-12 met-28 proA trpCDE*	17
PAO2198	*met-9020 catA1 chu-9002 trpAB lys-9015 nar-9011 leu-9014 argG9036*	H. Matsumoto, Shinshu University, Matsumoto, Japan
PAO944	*thr-9011 cys-54 pur-67*	32
PAO236.313	*ilvBC trpCDE pro-82 argF10 leu-10 str-1*	PAO236 × PAO25, sp. mut., *str-1*
Plasmids		
R68.45	Tc^r Km^r Cb^r Cma	15
pRO1614	Tc^r Cb^r	30
pRO1727	Tc^r Cb^r	11
pRO1769	Sm^r Gm^r	10
pRO1853	Cb^r	This study
pRO1862	Tc^r	This study
pRO1863	Tc^r	This study
pRO1877		This study
pRO1887	Tc^r	This study
pRO1892	Gm^r	This study

[a] Gene designations are as previously described (11, 17) except for the new gene designations *tynC1*, *tynD1*, *tynD1*, and *tynE1*, which are described in the text. Plasmids encode resistance to tetracycline (Tc), kanamycin (Km), carbenicillin (Cb), streptomycin (Sm), or gentamicin, kanamycin, and tobramycin (Gm). Cma, Chromosome mobilizing ability.

[b] Abbreviations: NTG, nitrosoguanidine mutagenesis; Tya$^-$, inability to utilize tyramine as the sole source of carbon; sp. mut., spontaneous mutation. Strain PAO236.313 was constructed by mating PAO236 × PAO25 (R68.45).

HPA. This product is indicative of a catabolic pathway involving a *meta* ring cleavage enzyme. The yellow product had absorption maxima of equal intensity at 380 and 320 nm at pH 7.0. Upon shift to pH 7.5, the intensity of the peak at 380 nm was enhanced while the peak at 320 nm disappeared. These results were consistent with published reports (1, 5, 23) which indicated that the yellow product formed from HPA was MSA. The enzyme mediating this cleavage is HPADO.

TABLE 2. Growth of wild-type and mutant strains of *P. aeruginosa* on aromatic amines and putative catabolic pathway intermediates

Strain	Relevant description or genotype[a]	Growth on[b]:						
		TYA	OCA	DOP	NOR	4HPAA	HPA	HGA
PAO1	Prototroph	+	+	+	+	+	+	+
PAO1.221	*tynC1*	−	−	+	+	−	+	+
PAO1.303	*tynD1*	−	−	+	+	−	+	+
PAO1.197	*tynE1*	−	−	−	−	−	−	+
PAO1.185	*tynF1*	−	−	−	−	−	−	+

[a] Genotypes are described in the text. Strains PAO1.221 (*tynC1*) and PAO1.303 (*tynD1*) are strains with identical phenotypes but with mutations in different loci (see text). Strains PAO1.197 (*tynE1*) and PAO1.185 (*tynF1*) also have identical growth properties but are distinguished by the accumulation of a yellow intermediate in the growth medium of the latter but not of the former strain when grown on any of the above aromatic compounds (except HGA). That the mutations in the two strains are in different loci is also confirmed by enzymatic analysis (see text).

[b] Strains were grown on solid MMO minimal medium supplemented with 0.2% tyramine (TYA), octopamine (OCA), dopamine (DOP), norepinephrine (NOR), 4HPAA, HPA, or HGA. Growth (+) or no growth (−) was scored after 1 and 2 days of incubation at 37°C.

Soluble cell extracts from wild-type cells induced with tyramine, octopamine, dopamine, norepinephrine, 4HPAA, or HPA showed high levels of HPADO activity (Table 3). Of these, relatively lower levels were seen in extracts of cells grown with tyramine or octopamine when compared with the levels in extracts of cells grown with the other substrates. This may reflect the increased number of enzymatic steps involved in the conversion of tyramine and octopamine to inducer or substrate compared with that for the conversion of the latter compounds (Fig. 1; Table 2). Extracts of cells induced with HPA had an HPADO activity that was approximately fourfold higher than the activities of cells induced with tyramine or octopamine. Cells grown on lactate alone produced no detectable HPADO activity. No HPADO activity could be found in cell extracts of mutant strain PAO1.197 (*tynE1*) induced with tyramine, 4HPAA, or HPA (Table 3), confirming that the *tynE1* mutation results in HPADO deficiency.

In contrast to the activities in cell extracts from wild-type cells, HPADO activities in extracts from strains deficient in a putative 4HPAA hydroxylase, PAO1.221 (*tynC1*) and PAO1.303 (*tynD1*), are induced by HPA but not by tyramine or 4HPAA (Table 3). However, strain PAO1.185 (*tynF1*), which lacks the enzyme for catabolism of the ring cleavage product (see below), produced HPADO activity under all inducing conditions. These data indicated that the inducer of HPADO activity was probably HPA and/or the semialdehyde ring cleavage product.

The addition of NAD to reaction mixtures containing MSA generated with soluble extract from 4HPAA-induced wild-type cells caused a rapid disappearance of the absorbance at 380 nm. This suggested that the semialdehyde is

TABLE 3. HPADO activity of wild-type and mutant cells of *P. aeruginosa* PAO1

Strain	Relevant description or genotype[a]	Growth conditions[b]	HPADO activity[c]
PAO1	Prototroph	LCT	<1[d]
		LCT + TYA	610
		LCT + OCA	420
		LCT + DOP	1080
		LCT + NOR	1040
		LCT + 4HPAA	950
		LCT + HPA	2060
PAO1.221	*tynC1*	LCT + 4HPAA	3
		LCT + HPA	2400
PAO1.303	*tynD1*	LCT + 4HPAA	<1
		LCT + HPA	1280
PAO1.197	*tynE1*	LCT	<1
		LCT + TYA	<1
		LCT + HPA	<1
PAO1.185	*tynF1*	LCT + TYA	490
		LCT + HPA	570

[a] Genotype designations are explained in the text.
[b] Strains were grown in MMO salts medium with 0.2% lactate (LCT) supplemented with 0.1% tyramine (TYA), octopamine (OCA), dopamine (DOP), norepinephrine (NOR), 4HPAA, or HPA where indicated. See the text for additional details.
[c] Activities of HPADO are expressed as milli-international units per milligram of protein and are the averages of at least two independent measurements.
[d] No detectable activity.

metabolized by MSADH, as is seen with 4HPAA-induced cell extracts of *Pseudomonas putida* U (35). Storage of the *P. aeruginosa* soluble extract overnight at 4°C caused a loss of MSADH activity which was restored by the addition of dithiothreitol (final concentration, 2 mM). Soluble extracts from 4HPAA-induced cells of strain PAO1.185 (*tynF1*) showed no MSADH activity, confirming that the *tynF1* mutation causes a deficiency in MSADH activity.

Gene cloning. Plasmid pRO1853 contains approximately 7.3 kilobases (kb) of PAO1 chromosomal DNA inserted into the unique *Bam*HI site in the tetracycline resistance locus of the vector pRO1614 (30). The recombinant plasmid complemented the *tynD1*, *tynE1*, and *tynF1* mutations in strains PAO1.303, PAO1.197, and PAO1.185, respectively (Fig. 2) but not the *tynC1* mutation in strain PAO1.221. A deletion derivative, pRO1877, prepared by *Pst*I cleavage and religation, located the DNA complementing the *tynE1* and *tynF1* mutations to the distal 6.0-kb portion of the plasmid (as drawn). A 1.1-kb portion of the vector coding for carbenicillin resistance was also deleted, so the only possible selection was for complementation of mutant phenotypes. Plasmid pRO1877 did not complement the *tynD1* mutation. The internal 3.0-kb *Eco*RI fragment of pRO1853 (and pRO1877) was subcloned into the *Eco*RI site of plasmid pRO1769 (10), inactivating the streptomycin resistance locus. The resultant plasmid, pRO1892, also complemented the *tynE1* and *tynF1* mutations. Portions of pRO1853 were subcloned into the unique *Pst*I site of the vector pRO1727, inactivating the carbenicillin resistance locus. Plasmids pRO1862 and pRO1863 (each containing approximately 1.1 kb of vector DNA from pRO1853 in addition to subcloned chromosomal DNA) were isolated. Plasmid pRO1863, but not pRO1862, complemented the *tynD1* mutation. Neither of these latter plasmids complemented the *tynE1* or *tynF1* mutation (Fig. 2).

Plasmid pRO1887 contains 9.2 kb of PAO1 chromosomal DNA partially cleaved with *Pst*I inserted in the *Pst*I site of the vector pRO1727. This plasmid complemented the *tynC1* mutation in strain PAO1.221 only (Fig. 2). Restriction analysis of plasmids pRO1853 and pRO1887 demonstrated that these were different plasmids with no overlapping regions of cloned chromosomal DNA.

The effects of pRO1853 and plasmids derived from it on HPADO activity in 4HPAA-induced and noninduced transformed cells are shown in Table 4. Cell extracts of HPADO-deficient mutant strain PAO1.197 (*tynE1*) showed enzyme

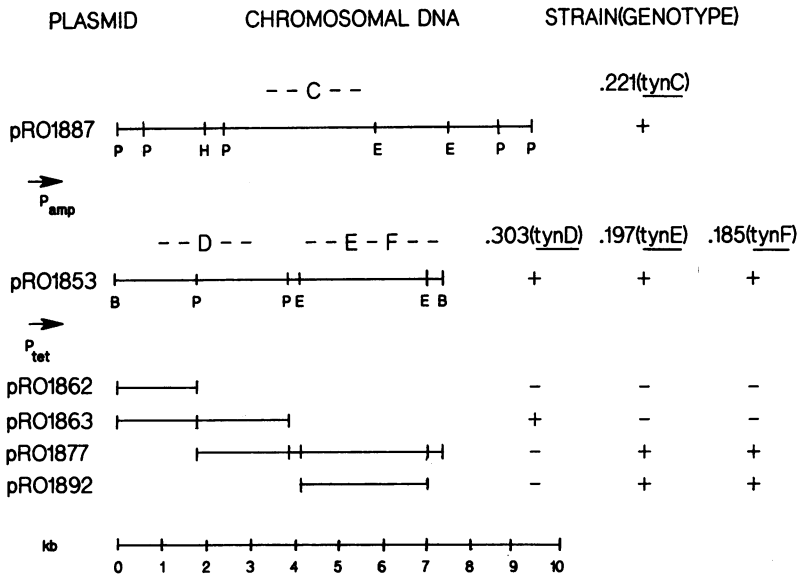

FIG. 2. Restriction endonuclease map of *P. aeruginosa* PAO1 chromosomal DNA fragments which complement various *tyn* mutations. Details of the plasmid constructions are provided in the text. The ability (+) or inability (−) of the plasmids to complement the various *tyn* mutations is indicated to the right of the restriction maps. Abbreviations: B, *Bam*HI; E, *Eco*RI; P, *Pst*I. Known vector promoters (30) are shown as arrows. The tetracycline promoter P_{tet} is present in pRO1853, pRO1862, and pRO1863 but not in pRO1877. The orientation of the insert in pRO1892 with respect to that of vector promoters is not known.

TABLE 4. HPADO activities in transformed wild-type and mutant cells of *P. aeruginosa*

Strain (plasmid)	Relevant description or genotype[a]	HPADO activity[b]	
		LCT	LCT + 4HPAA
PAO1	Prototroph	<1[c]	950
PAO1(pRO1853)		70	3490
PAO1(pRO1862)		<1	680
PAO1(pRO1863)		6	660
PAO1(pRO1892)		360	1050
PAO1.197	*tynE1*	<1	<1
PAO1.197(pRO1853)		60	870
PAO1.197(pRO1862)		<1	<1
PAO1.197(pRO1863)		<1	<1
PAO1.197(pRO1877)		50	250
PAO1.197(pRO1892)		360	540

[a] Genotypes are described in the text.
[b] HPADO activities are expressed as milli-international units of protein and are the averages of at least two independent measurements. Cells were grown in MMO salts medium with 0.2% lactate (LCT) supplemented with 0.1% 4HPAA when indicated. See the text for additional details.
[c] No detectable activity.

activity when the strain was transformed with plasmid pRO1853, pRO1877, or pRO1892. High HPADO activities were seen in extracts of noninduced cells transformed with the plasmids indicated above. The presence of pRO1892 caused a five- to sevenfold increase in this apparent constitutive activity when compared with that of cells transformed with pRO1853 (and pRO1877 transformed into PAO1.197). Low but reproducibly detectable apparent constitutive HPADO activity was seen in wild-type cells transformed with pRO1863. However, neither this plasmid nor pRO1862 restored HPADO activity to PAO1.197.

Chromosomal mapping. With the chromosome-mobilizing plasmid R68.45, the *tynC1*, *tynD1*, *tynE1*, and *tynF1* mutations were mapped in a cluster at about 40 min on the PAO1 chromosome map (Fig. 3). The *tynD1*, *tynE1*, and *tynF1* mutations were shown to be tightly linked by their presence on a single *Bam*HI fragment. Consequently, mapping experiments were performed only with strains PAO1.221 (*tynC1*) and PAO1.197 (*tynE1*). The respective coconjugal transfer frequencies of the two mutations showed they were tightly linked to the *proA* (0.89 and 0.84), *thr-48* (0.68 and 0.87), *lys-9015* (0.67 and 0.44), *argF* (0.84 and 0.62), and *argG* (0.54 and 0.35) mutations (Fig. 3). No linkage was seen between the *tynCDEF* gene cluster and mutations outside the 35-to-45-min region of the PAO1 chromosome (17).

DISCUSSION

P. aeruginosa PAO1 utilizes aromatic, biogenic amines as the sole sources of carbon and/or nitrogen, and initial catabolism of tyramine and octopamine converges at 4HPAL, which is converted to 4HPAA by soluble MSADH (11). Through the use of biochemical, enzymatic, and genetic analyses, we demonstrated that the catabolism of 4HPAA proceeds through HPA, which is cleaved between the second and third ring carbon atoms to form MSA. Similar analyses showed that amines hydroxylated at the third ring carbon (dopamine, norepinephrine, etc.) were also metabolized through HPA but that metabolism was not dependent on the presence of the proposed 4HPAA 3-hydroxylase activity. Mutants deficient in enzymes of each step of the pathway were isolated and characterized.

Previous reports demonstrated that a pyridine nucleotide-dependent hydroxylase was involved in the conversion of 4HPAA to HPA (1, 2, 8). Our failure to detect this activity could be due to the need for different cofactors or reaction conditions or to the fact that the enzyme is not stable under the cell disruption or assay conditions we used. Several 4-hydroxybenzoate-3-hydroxylases isolated from various *Pseudomonas* strains are unstable (38). Nevertheless, mutants PAO1.221 and PAO1.303 are probably deficient in the enzyme that mediates the conversion of 4HPAA to HPA. Cultures of these strains grown on tyramine accumulated 4HPAA in the growth medium. Both strains produced wild-type activities of the next enzyme in the pathway, HPADO, when they were induced with HPA but not when they were induced with 4HPAA. Cloning analysis showed that plasmid pRO1887 complemented the *tynC1* mutation in strain PAO1.221 but not the *tynD1* mutation in strain PAO1.303. The converse was true for plasmid pRO1863. This result indicated that there were two separate mutations causing deficiencies in the proposed 4HPAA 3-hydroxylase activity. The partially purified enzyme from *Pseudomonas ovalis* has

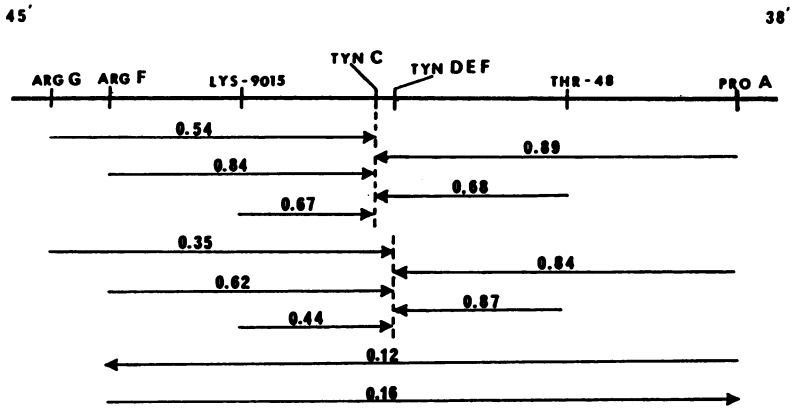

FIG. 3. Conjugational linkage map of the 35-to-45-min region of the *P. aeruginosa* PAO1 chromosome. The numbers represent the frequencies of coconjugal transfer of two markers by using the chromosome-mobilizing plasmid R68.45. Arrowheads point to the unselected marker. The map represents the composite of several independent experiments in which, for each linkage, 400 to 600 transconjugants were scored for the presence of the unselected marker. The locations of the *proA*, *thr-48*, *lys-9015*, *argF*, and *argG* markers have been previously determined (17).

two subunits (1), and it is possible that the two different mutations in strains PAO1.221 and PAO1.303 represent lesions in different subunit structural genes. Alternatively, one mutation could lie in a regulatory locus.

Results presented here show that catabolism of 4HPAA was through HPA and not through another possible intermediate, HGA. The enzyme mediating ring cleavage was HPADO, as seen by the accumulation of a yellow compound whose spectral properties were identical to those previously reported for the 2,3-cleavage product, MSA (5). This enzyme has been reported to be involved in the catabolism of HPA (1, 5, 35) and chlorinated derivatives (22, 26) in other pseudomonads. Enzyme induction studies in mutant strains indicated that HPADO was induced by HPA and/or its metabolic product, MSA. HPA is the inducer for HPADO and the enzymes subsequently involved in the catabolism of MSA to tricarboxylic acid cycle intermediates in *P. putida* P23X (3).

Growth studies showed that all of the aromatic amines studied in this and the previous work (11) were catabolized through HPA when it was present as the sole source of carbon or nitrogen. However, wild-type strains grown on octopamine as the sole source of carbon and nitrogen failed to produce detectable HPADO activity (data not shown). High HPADO levels were seen in PAO1 cells grown on octopamine as the sole carbon source (with added ammonia) or the sole nitrogen source (with added lactate). In addition, strain PAO1.197, deficient in HPADO, utilized octopamine as the sole source of carbon and nitrogen but not as the sole source of carbon or nitrogen (data not shown). Mutants deficient in all of the other ring cleavage enzymes known to be produced by strain PAO1, catechol 1,2-dioxygenase (29), protocatechuate 3,4-dioxygenase (28), homogentisate 1,2-dioxygenase (29), and gentisate 1,2-dioxygenase (R. H. Olsen, unpublished results), all grow on octopamine as the sole source of carbon and nitrogen. These data suggest that there is another ring cleavage pathway in strain PAO1 responsible for catabolism of octopamine as the sole source of carbon and nitrogen. Different cleavage pathways for structurally related aromatic compounds are not unknown. For example, *Pseudomonas* species strain CBS3 metabolizes 4HPAA through HGA, and it metabolizes 4-chlorophenylacetate through HPA (22).

As *meta* fission pathways were discovered, one view was that genes for extradiol (*meta*) cleavage enzymes were usually encoded on catabolic plasmids, while intradiol (*ortho*) ring cleavage enzyme genes were mainly located on the chromosome. This view, however, is no longer tenable. For example, Sinclair et al. (33) have recently demonstrated in a strain of *P. putida* the chromosomal location of genes encoding a TOL-like pathway with extensive homology with the TOL plasmid pWWO. We have reported in the present study that a mutation which caused a deficiency in the *meta* cleavage enzyme HPADO was located on the PAO1 chromosome. These results show that *meta* cleavage pathways are also chromosomally encoded. Indeed, all known bacterial catabolism of HPA is through a *meta* cleavage pathway (12). Since this catabolic trait is not known to be plasmid associated, chromosomal location of *meta* cleavage pathways may not be unusual.

Transformation of the HPADO-deficient mutant PAO 1.197 (*tynE1*) with plasmid pRO1853 or subclones derived from it (pRO1877 and pRO1892) restored HPADO activity to the mutant cell extracts. Transformed cells also showed high apparent constitutive HPADO activity. The recombinant plasmids are present at a copy number of about 35 to 40 per cell. Therefore, the high noninduced activity may have been the result of a cumulative basal level expression from 35 to 40 gene copies. However, part of the activity may be the result of a repressor titration phenomenon (19) in which a limited number of chromosomally encoded repressor molecules are bound to an increased number of cloned repressor-binding sites. This can result in apparent constitutive expression of the activities of enzymes whose syntheses are under control of the repressor (19). Repressor titration is suggested here by the HPADO activities from cells transformed with pRO1863. This plasmid does not contain the HPADO structural gene, but low enzyme levels were seen in noninduced, wild-type cell extracts. This result suggests that a possible repressor-binding site is located between the *Pst*I sites at 1.9 and 4.1 kb on DNA that does not encode the HPADO structural gene. Cells transformed with pRO1892, which contains the HPADO structural gene but lacks the proposed repressor binding site, showed a five- to sevenfold increase in apparent constitutive HPADO activity compared with that of cells transformed with chimeric plasmids containing the proposed binding site as well as the HPADO structural gene (pRO1853 and pRO1877). In pRO1892-transformed cells, low basal level expression from the 35 to 40 closed copies of the structural gene may not be hindered by a proposed binding of repressor molecules, yielding a higher apparent constitutive activity.

Further catabolism of the muconic semialdehyde derivative is mediated by MSADH. This activity was present in extracts of wild-type cells but absent in mutant strain PAO1.185. The product of this enzyme was probably 2-hydroxy-5-carboxymethylmuconic acid, as was seen with the enzyme from *P. putida* U (35). Due to the unavailability of the substrate and its instability, we did not attempt to quantitate MSADH levels in mutant and wild-type cells. The pathway of 4HPAA catabolism through the semialdehyde is identical to that previously reported for other strains (8, 35) and probably proceeds to pyruvate and succinic semialdehyde. We have not investigated the later portions of the pathway in strain PAO1.

The *tynCDEF* genes were shown to be clustered at about 40 min on the PAO1 chromosomal map (17) by using the chromosome-mobilizing plasmid R68.45. Genes responsible for the initial catabolism of tyramine to 4HPAL map in a different chromosomal region, at about 70 min on the PAO1 map (11). These results illustrate an apparent feature of gene organization in *Pseudomonas* spp. Genes encoding functions of a particular catabolic pathway are often clustered (9, 25, 29), but, in contrast to previously held ideas, separate clusters may be present in all regions of the chromosome (14).

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