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Initial Catabolism of Aromatic Biogenic Amines by *Pseudomonas aeruginosa* PAO: Pathway Description, Mapping of Mutations, and Cloning of Essential Genes

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Pseudomonas aeruginosa **PAO1 was able to utilize several aromatic biogenic amines as sole sources of carbon or nitrogen. These included the phenethylamines tyramine and dopamine and the phenethanolamines octopamine, synephrine, and norepinephrine. Initial catabolism of the phenethylamines was mediated by a membrane-bound tyramine dehydrogenase which produced 4-hydroxyphenylacetaldehyde (4HPAL) with tyramine as the substrate. The enzyme was induced by growth with both classes of amines. Initial catabolism of octopamine (except when present as the sole source of carbon and nitrogen) was mediated by a soluble enzyme with activity against the phenethanolamines but not against tyramine or dopamine. The product of the reaction with octopamine as substrate was also 4HPAL. Addition of NAD to reaction mixtures yielded 4-hydroxyphenylacetic acid and NADH. These activities, octopamine hydrolyase and 4-HPAL dehydrogenase (measured as a combined activity, OCAH-4HPALDH), were only induced by growth with phenethanolamines. However, the combined activities were not observed in extracts from cells grown with octopamine as the sole source of carbon and nitrogen, suggesting that an alternate pathway is used under this growth condition. Two independently isolated mutant strains were unable to utilize tyramine as a sole source of carbon or nitrogen. These mutants were also unable to utilize dopamine but grew at wild-type rates on the phenethanolamines. The mutations were mapped at about 70 min on the PAO1 chromosome with the chromosome-mobilizing plasmid R68.45, and both were linked to the *catA1*, *mtu-9002*, *tyu-9009*, and *puuE* mutations. DNA complementing both of the mutations was cloned on a single *Bam*HI fragment approximately 13.8 kilobase pairs in length. Analysis of a subcloned fragment showed that the two mutations were in different genes.**

Aromatic amines such as tyramine, dopamine, norepinephrine, and others (Fig. 1) are present in natural environments; many are widely recognized as essential components of mammalian nervous systems. These compounds can be divided into two classes, phenethylamines and phenethanolamines, based on hydroxyl substitution of the amine-containing side chain. Bacterial catabolism of the phenethylamines tyramine and dopamine has been better characterized than that of the phenethanolamines. Catabolism of phenethylamines is initiated by an oxidative deamination of the primary amine-containing side chain, which, with tyramine as a substrate, produces 4-hydroxyphenylacetaldehyde (4HPAL) and ammonia. This is most often accomplished through the action of a monoamine (tyramine) oxidase. Tyramine oxidase has been studied in several bacteria, particularly *Klebsiella aerogenes* (25), *Salmonella typhimurium* (23), *Sarcina lutea* (34, 35), and a strain of *Pseudomonas aeruginosa* (21), and has been implicated in the tyramine catabolism of a wide range of organisms (22). Alternatively, an aromatic amine dehydrogenase with high activity against tyramine and dopamine which requires cytochrome *c* or an artificial electron acceptor for catalytic activity has been described for a *Pseudomonas* strain (19). Another amine dehydrogenase active against aliphatic and aromatic amines with activity against tyramine has been described for a strain of *Pseudomonas putida* (8–10), although the organism does not utilize tyramine as a sole source of carbon (8). The route of further catabolism of 4HPAL has not been well studied but may proceed through 4-hydroxyphenylacetic acid (4HPAA), followed by conversion to the aromatic ring cleavage substrates 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid [HPA]) (6, 29) or 2,5-dihydroxyphenylacetic acid (homogentisic acid) (4, 15).

Bacterial catabolism of the phenethanolamines octopamine, synephrine, norepinephrine, and epinephrine has been less well studied. The tyramine oxidases of *K. aerogenes* (25) and *P. aeruginosa* (21) but not *S. lutea* (35) are active against several phenethanolamines, but the products of the reactions and the routes of further catabolism were not determined. The most extensive investigation of bacterial degradation of the phenethanolamines was performed with *Arthrobacter synephrinum* (1, 20, 32). Catabolism is initiated by an enzyme tentatively designated synephrine hydrolyase, which is specific for synephrine. The products of the oxygen-independent reaction are 4HPAL and methylamine. Further catabolism of the aromatic moiety occurs through 4HPAA and HPA, which undergoes *meta* cleavage to form 2-hydroxy-5-carboxymethylmuconate semialdehyde (20, 32).

In this report we describe the pathways used by *P. aeruginosa* PAO1 for the initial catabolism of a representative phenethylamine (tyramine) and phenethanolamine (octopamine). We also describe mutant strains unable to

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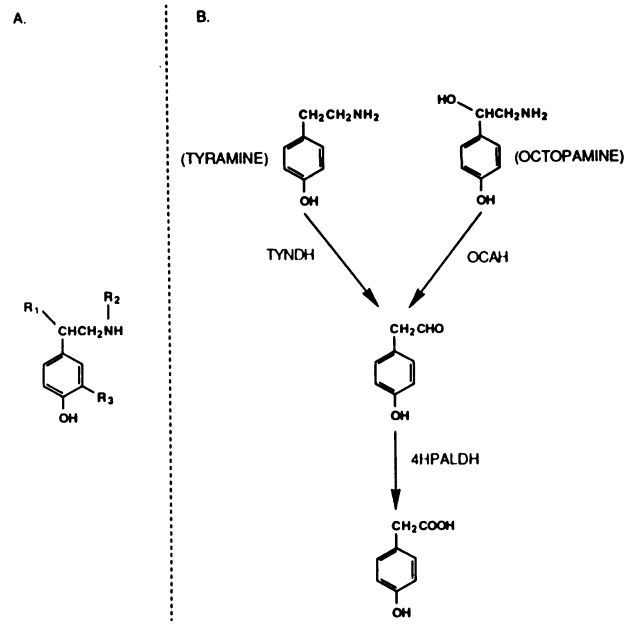

FIG. 1. (A) Aromatic biogenic amines used in this study and (B) proposed pathway of initial catabolism of tyramine and octopamine in *P. aeruginosa* PAO1. Amines: tyramine—R_1, R_2, and R_3 = H; octopamine—R_1 = OH, R_2 and R_3 = H; dopamine—R_1 and R_2 = H, R_3 = OH; synephrine—R_1 = OH, R_2 = CH_3, R_3 = H; epinephrine—R_1 and R_3 = OH, R_2 = CH_3; norepinephrine—R_1 and R_3 = OH, R_2 = H.

catabolize these compounds and map and clone portions of the PAO1 chromosome which complement these mutations.

(Portions of this work have been presented elsewhere [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, plasmids, and growth media. The bacterial strains and plasmids used in these studies are listed in Table 1. All bacterial mutants were derived from *P. aeruginosa* PAO1 (16). Bacteria were grown at 37°C in a previously described basal salts medium (MMO) (31). When an aromatic amine was the sole source of nitrogen, Burk's nitrogen-free medium (27) was used. A concentrated stock solution of lactic acid was sterilized separately by autoclaving and added to sterile salts at a final concentration of 0.2% (wt/vol) where noted. Aromatic compounds were added directly to sterile salts at a final concentration of 0.1% (wt/vol) unless they were present as the sole source of carbon, when the final concentration was 0.2% (wt/vol). Amino acid supplements were added to final concentrations of 0.5 mM. Complex medium (TNA) consisted of the following (in grams per liter): tryptone, 10; yeast extract, 5; NaCl, 1; proteose peptone, 1; potassium nitrate, 0.5; and calcium chloride, 0.5. When indicated, carbenicillin (500 μg/ml), tetracycline (50 μg/ml), or kanamycin (600 μg/ml) was added to complex medium (without calcium chloride). All solid media contained 1.5% (wt/vol) agar.

Mutagenesis with nitrosoguanidine was performed by standard techniques. Survivors growing on minimal medium with or without supplemental amino acids were tested by plating on the appropriate medium with an aromatic amine as the sole source of carbon or nitrogen or both. Presumptive mutants were purified by single-colony isolation and retested on medium with various aromatic compounds as the sole source of carbon. Mutants also unable to grow on a carbohydrate as the sole source of carbon were discarded.

Extract preparation and enzyme assays. Cell extracts used in enzyme assays were prepared from cells cultured in 100 ml of the appropriate salts medium with or without added carbohydrate carbon source, ammonium sulfate, and aromatic compound as inducer, carbon, or nitrogen source. Cultures were inoculated with a 20-fold dilution of starter cultures grown overnight in MMO medium supplemented with 0.2% (wt/vol) lactate. Late-exponential-phase cells were harvested by centrifugation (8,000 × *g*, 10 min), washed once in carbon-free MMO, and frozen at −70°C until needed. Frozen cell pellets were resuspended in 5 ml of 0.1 M Tris hydrochloride (pH 7.5) and ruptured by treatment with a probe-type ultrasonic oscillator (18). The soluble supernatant fraction of ruptured cells was collected after centrifugation (105,000 × *g* for 2 h at 4°C) and used in assays for the combined octopamine hydrolyase-4HPAL dehydrogenase (OCAH-4HPALDH) activities. The membrane-enriched particulate fraction was homogenized in the above buffer and recentrifuged. The washed membrane fraction was suspended in 2.0 ml of buffer and used as the source of tyramine dehydrogenase (TYNDH).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype[a]	Derivation or source (reference)[b]
P. aeruginosa		
PAO1	Prototroph	Laboratory stock (16)
PAO1.132	*vma-1*	PAO1; sp. mut.; fast vma (this study)
PAO1.299	*vma-1 tynA1*	PAO1.132; NTG; Tya^- (this study)
PAO1.222	*vma-1 tynB1*	PAO1.132; NTG; Tya^- (this study)
PAO944	*thr-9011 cys-54 pur-67*	28
PAO2198	*met-9020 catA1 chu-9002 trpAB lys-9015 nar-9011 leu-9014 arg-9036*	Matsumoto[c]
PAO2324	*met-9020 catA1 tyu-9009 nar-9011 puuD6*	Matsumoto
PAO2375	*met-9020 catA1 nar-9011 mtuD9002*	Matsumoto
PAO4032	*met-9020 catA1 nar-9011 mtuD9002 dcu-9013*	Matsumoto
Plasmids		
R68.45	Tc^r Km^r Cb^r Cma	13
pRO1614	Tc^r Cb^r	26
pRO1727	Tc^r Cb^r	This study
pRO1865	Cb^r	This study
pRO1914	Tc^r	This study

[a] Gene designations are as described previously (16) except for the new gene designations *tynA* and *tynB*, which are described in this report and are adopted from the nomenclature used for *Klebsiella* and *Salmonella* spp. (24). The *vma-1* mutation is an uncharacterized mutation allowing fast growth on vanillylmandelic acid. Tc^r, Km^r, Cb^r, Resistance to tetrycycline, kanamycin, and carbenicillin, respectively; Cma, chromosome-mobilizing ability.

[b] Abbreviations: sp. mut., spontaneous mutation; fast vma, fast growth on solid medium with vanillylmandelic acid as the sole source of carbon; NTG, nitrosoguanidine mutagenesis; Tya, tyramine utilization as the sole source of carbon or nitrogen.

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Enzyme activities were determined spectrophotometrically and are reported as nanomoles of product formed per minute per milligram of protein. TYNDH activity was determined from reduction of the dye 2,6-dichlorophenolindophenol (DCIP) (2). Reaction mixtures containing 100 mM Tris hydrochloride, pH 7.5, 2 mM KCN, various amounts of washed membrane-enriched particulate fractions, and 0.16 mM DCIP were started by the addition of substrate (final concentration, 10 mM). Activity was measured as a decrease in absorbance at 600 nm and was quantitated from an assumed DCIP extinction coefficient of 21.5×10^3U (2). Addition of 5 mM phenazine methosulfate caused a significant loss of activity. The combined OCAH-4HPALDH activities were determined by following the increase in absorbance at 340 nm from the formation of NADH. Reaction mixtures contained 100 mM Tris hydrochloride, pH 7.5, 0.5 mM NAD, and various amounts of soluble cell extract and were initiated by the addition of 20 mM substrate (final concentration). Protein was measured by the method of Bradford (5).

Experiments to determine whether soluble and membrane fractions from induced cells were active against octopamine or tyramine, respectively, in the absence of oxygen were performed in Warburg vessels overnight at 37°C with shaking. Buffer, the source of enzyme, and cofactors were placed in the bottom of the flask, and substrate was placed in the side arm. After being flushed with nitrogen for at least 10 min, the flasks were sealed from the atmosphere, and the contents of the side arm were tipped into the flask. A control flask containing HPA as the substrate and the soluble extract of cells induced by growth with tyramine or octopamine was included. *P. aeruginosa* metabolizes both tyramine and octopamine through *meta* cleavage of HPA (Cuskey and Olsen, manuscript in preparation), the product of which is bright yellow. As this reaction requires molecular oxygen, the absence of a yellow color indicated that the internal atmosphere was anaerobic. After exposure of the flask contents to air, a bright yellow color developed within minutes.

Analytical methods. Aromatic amines and catabolic pathway intermediates were chromatographed by using inert plastic sheets precoated with cellulose containing a fluorescent indicator (Eastman Kodak Co., Rochester, N.Y.). The solvent system was composed of 1-butanol, ethanol, and 3% ammonium hydroxide (80:22:38). Compounds were visualized under UV light. Additionally, phenolic compounds were visualized after spraying the dried chromatograms with a 1:1 solution of ferric chloride (0.05 M) and potassium ferricyanide (0.05 M) (30), and aldehydes were visualized by spraying the dried chromatograms with 2,4-dinitrophenylhydrazine (0.4% in 2 N HCl) (30).

High-pressure liquid chromatographic (HPLC) analysis was performed with a Whatman (Hillsboro, Oreg.) ODS-2 Partisil C-18 column (25 by 0.4 cm) with a Beckman (Fullerton, Calif.) HPLC. The solvent systems were water-acetic acid, 99.5:0.5 (solvent A), or water-methanol-acetic acid, 49.5:50:0.5 (solvent B). At a flow rate of 1.5 ml/min, the solvents were applied in the following manner after sample injection; 5 min of solvent A, 25 min of a linear gradient from 100% A to 100% B, 5 min of solvent B, and 5 min of a linear gradient from 100% B to 100% A (Lute and Olsen, manuscript in preparation).

The R_fs (tyramine, 0.76; octopamine, 0.71; 4HPAL, 0.88; and 4HPAA, 0.58) and retention times (tyramine, 14.6 min; octopamine, 5.3 min; and 4HPAA, 22.8 min) of authentic aromatic compounds and potential catabolic pathway intermediates by the above chromatographic techniques were identical.

4HPAL was synthesized by the method of Auterhof and Roth (3). Alternatively, preparation of the aldehyde proceded via a two-step synthesis beginning with 2(*p*-methoxyphenyl)ethanol. To 1 mmol of 2(*p*-methoxyphenyl) ethanol in cold water was added 33 μmol of sodium dichromate. The reaction mixture turned blue-green after 30 min, at which time the mixture was extracted with diethyl ether. The aldehyde was recovered by flash evaporation of the ethereal layer. The methoxy-protecting group was removed by using boron tribromide in methylene chloride, and 0.8 mmol of *p*-methoxyphenylacetaldehyde was dissolved in 50 ml of methylene chloride. To this ice-cold solution was added, dropwise with stirring, 15 ml of a solution of boron tribromide (3 mmol) in methylene chloride. The solution was slowly warmed to room temperature and allowed to react for 12 h. Water was then added dropwise to the reaction mixture. The hydrolyzed product was reacted with sodium sulfite, extracted with chloroform, and reacidified. Flash evaporation of the aqueous layer resulted in accumulation of the desired product, with a melting point (uncorrected) of 181°C. The melting point (uncorrected) of the 2,4-dinitrophenylhydrazine derivative was determined and agreed with the published value (183 to 184°C) given by Auterhof and Roth (3).

Genetic techniques. Plasmids pRO1614 (26) and pRO1727 were used as cloning vectors in these experiments. Plasmid pRO1727 was derived from pRO1614 by deleting approximately 1.6 kilobase pairs (kb), including one of the two *PstI* sites. The remaining *PstI* site was in the gene determining resistance to carbenicillin, allowing insertional inactivation of this gene (Olsen, unpublished data).

Techniques for DNA isolation and purification (26), restriction endonuclease cleavage and ligation of purified DNA (7), bacterial cell transformations (26), rapid cell lysis for visualization of recombinant plasmids (11), and agarose gel electrophoresis (7) have been described.

Chromosomal mapping experiments involving mutants unable to utilize tyramine were performed with the chromosome-mobilizing plasmid R68.45 by standard techniques (13).

Chemicals and reagents. Pyridine nucleotides, enzyme substrates, aromatic compounds, and other reagents used in growth experiments, enzyme induction, and enzyme assays were of the highest purity commercially available from Sigma Chemical Co., St. Louis, Mo., or Aldrich Chemical Co., Milwaukee, Wis. Antibiotics were purchased from Sigma. Other chemicals were of reagent grade purity, purchased from Fisher Scientific Co., Pittsburgh, Pa., or Sigma. Tryptone, yeast extract, proteose peptone, and agar were purchased from Difco Laboratories, Detroit, Mich.

Restriction endonucleases and other enzymes involved with DNA manipulations were purchased from International Biotechnologies Inc. (New Haven, Conn.) or Bethesda Research Laboratories (Gaithersburg, Md.).

RESULTS

Growth. *P. aeruginosa* PAO1 utilized tyramine, dopamine, octopamine, and norepinephrine on solid media as sole sources of carbon or nitrogen (not shown). Strain PAO1 also utilized synephrine as a carbon source but grew poorly, if at all, on this substrate as the sole source of nitrogen. This strain could not utilize epinephrine, 3,4-dihydroxyphenylalanine, or phenethylamine as sole sources of carbon

TABLE 2. Specific activities of enzymes of aromatic amine catabolism in wild-type and mutant strains of *P. aeruginosa*

Strain	Growth conditions[a]	Sp act (mIU/mg of protein)[b]	
		TYNDH	OCAH-4HPALDH
PAO1	Lct	<1	<1
	Lct + Amm + Tya	530	<1
	Lct + Amm + Oca	610	40
	Lct + Tya	800	<1
	Lct + Oca	960	40
	Amm + Tya	1,330	<1
	Amm + Oca	1,140	30
	Tya	710	<1
	Oca	<1	<1
PAO1(pRO1865)	Lct	<1	<1
	Lct + Amm + Tya	2,550	<1
	Lct + Amm + Oca	2,830	70
PAO1(pRO1914)	Lct	<1	<1
	Lct + Amm + Tya	710	<1
PAO1.299(*tynA1*)	Lct	<1	<1
	Lct + Amm + Tya	<1	<1
	Lct + Amm + Oca	<1	40
PAO1.299(pRO1865)	Lct	<1	<1
	Lct + Amm + Tya	2,190	<1
PAO1.299(pRO1914)	Lct	<1	<1
	Lct + Amm + Tya	<1	<1
	Amm + Tya	NG	NG
PAO1.222 (*tynB1*)	Lct	<1	<1
	Lct + Amm + Tya	<1	<1
	Lct + Amm + Oca	<1	40
PAO1.222(pRO1865)	Lct	<1	<1
	Lct + Amm + Tya	2,010	<1
PAO1.222(pRO1914)	Lct	<1	<1
	Lct + Amm + Tya	<1	<1
	Amm + Tya	620	<1

[a] When 0.1% ammonia was present as the nitrogen source (Amm), cells were grown in liquid MMO (31) medium. When an aromatic amine was used as the sole source of nitrogen, cells were grown in liquid Burk's nitrogen-free medium (27). Carbon and nitrogen sources included lactate (Lct), tyramine (Tya), and octopamine (Oca).

[b] A value of <1 indicates no detectable activity. NG, No growth.

or nitrogen under the growth conditions used in these studies. Because of their stability and ease of handling, we concentrated our initial studies on the catabolism of the biogenic amines with the phenethylamine tyramine and the phenethanolamine octopamine.

Cells grown in liquid culture on lactate and ammonia exhibited a lag of 3 to 10 h when transferred to fresh medium containing tyramine or octopamine as the sole source of carbon, nitrogen, or both. The shortest lag was when the cells were transferred to a medium in which the amines were the sole source of carbon only (with added ammonia), while the longest lags occurred when the cells utilized the amines as sole sources of both carbon and nitrogen. Approximate doubling times with tyramine and octopamine as the sole source of carbon (48 and 86 min, respectively), nitrogen (64 and 69 min, respectively), or both carbon and nitrogen (77 and 96 min, respectively) were greater than for cells growing on lactate and ammonia (38 min).

The washed membrane fraction from tyramine-grown wild-type cells aerobically or anaerobically converted tyramine to a product that reacted with 2,4-dinitrophenylhydrazine and which resembled authentic 4HPAL, as determined by chromatographic analysis. This fraction also reduced the dye DCIP in the presence of tyramine or dopamine. No reduction of DCIP was seen when using the soluble fraction. These data indicated that the first step in tyramine catabolism involved a membrane-bound TYNDH, and the product of this reaction appeared to be 4HPAL. Growth of strain PAO1 on lactate in the presence of tyramine or octopamine induced approximately equal levels of TYNDH (530 and 610 mIU/mg of protein, respectively) with tyramine as the substrate (Table 2). The induction of TYNDH by octopamine (and other phenethanolamines; data not shown) was surprising because the enzyme was active only against phenethylamines (data not shown).

The soluble extract of wild-type cells grown on lactate and octopamine converted octopamine to a compound which also reacted with 2,4-dinitrophenylhydrazine and which was indistinguishable from 4HPAL by chromatographic analysis. In the presence of octopamine and NAD (but not NADP), the soluble extract of cells grown on lactate and octopamine formed a compound which was indistinguishable from authentic 4HPAA, and the reactions were oxygen independent. These data indicated that initial catabolism of octopamine (except when it was present as the sole source of carbon and nitrogen, see below) was mediated by a soluble enzyme tentatively designated octopamine hydrolyase (OCAH). The product of the oxygen-independent reaction was 4HPAL, which is converted to 4HPAA by an NAD-dependent 4HPAL dehydrogenase (4HPALDH). A coupled reaction based on the reduction of NAD was developed. The combined activities (OCAH-4HPALDH) of both enzymes were required to reduce NAD in the presence of octopamine and were induced when PAO1 cells were grown on lactate with octopamine (40 mIU/mg of protein, Table 2). No activity was detected when the cells were grown with tyramine (Table 2), and no activity occurred with tyramine (or dopamine) as the substrate (data not shown). Cells grown on octopamine as the sole source of carbon and nitrogen did not exhibit OCAH-4HPALDH activity (Table 2).

Mutant isolation and characterization. Mutants unable to utilize tyramine were isolated after exposure of parent cells to nitrosoguanidine. Two separate classes of mutants (see below), represented by strains PAO1.299 and PAO1.222, were isolated which were unable to use tyramine (or dopamine) as a sole source of carbon or nitrogen (not shown). Both mutant strains utilized octopamine as well as the other phenethanolamines as a source of carbon or nitrogen or both (not shown). Neither mutant strain produced TYNDH (Table 2), although the combined OCAH-4HPALDH activity was present in the soluble fractions of cells induced by growth with octopamine.

Chromosomal mapping. With the chromosome-mobilizing plasmid R68.45, the mutations designated *tynA1* and *tynB1* (Table 1) of strains PAO1.299 and PAO1.222, respectively, were mapped to the late region of the *P. aeruginosa* PAO chromosome (Fig. 2). An additional putative mutation in strain PAO1.299 caused cell clumping, and this lowered the conjugation efficiency of these cells. Consequently, most mapping experiments were performed with strain PAO1.222. Both mutations were linked to the same region of the chromosome, however, by data from cloning experiments (see below). The *tynB1* mutation was tightly linked to *tyu-9009* and *mtu-9002* (0.66 and 0.87% coconjugation fre-

quency of transfer, respectively), both located at 70 to 75 min on the PAO chromosome map, and was less tightly linked to the *puuE* and *catA1* mutations (0.31 and 0.06, respectively). These data indicated that the *tynB1* mutation was located at about 70 to 75 min, between the *mtu-9002* and *tyu-9009* markers.

Cloning. Plasmid pRO1865 contained 13.5 kb of PAO1 chromosomal DNA inserted into the *Bam*HI site of the vector plasmid pRO1614. No additional *Bam*HI sites were present in this DNA fragment (Fig. 3). This plasmid complemented both the *tynA1* mutation in strain PAO1.299 and the *tynB1* mutation in strain PAO1.222 and allowed transformed cells to utilize tyramine (and dopamine) as a sole source of carbon or nitrogen. Transformed mutant cells also produced TYNDH (Table 2). Levels of TYNDH were higher in induced transformed mutant and wild-type cells than in induced nontransformed wild-type cells. No activity was seen in the membrane fractions of noninduced, transformed cells.

Plasmid pRO1914, which was insolated in a separate cloning experiment, contains 2.8 kb of PAO1 chromosomal DNA inserted into the *Pst*I site of the vector plasmid pRO1727, inactivating the carbenicillin resistance locus. There were no additional *Pst*I sites present within the insert (Fig. 3). Unlike plasmid pRO1865, plasmid pRO1914 complemented the *tynB1* mutation but not the *tynA1* mutation and allowed only transformed PAO1.222 cells to utilize tyramine and dopamine as sole sources of carbon or nitrogen. However, the presence of lactate in the growth medium appeared to repress the formation of TYNDH in transformed mutant cells; no enzyme activity was seen when PAO1.222(pRO1914) cells were grown on lactate plus ammonia and tyramine (Table 2). When the cells were grown on tyramine as the sole source of carbon, wild-type levels of TYNDH were seen in the membrane fractions. This relationship was not seen with wild-type cells transformed with pRO1914, i.e., PAO1(pRO1914) cells produced TYNDH under all inducing conditions tested (Table 2). Plasmids pRO1865 and pRO1914 contained overlapping sections of DNA, as seen from restriction endonuclease digests (Fig. 3).

DISCUSSION

P. aeruginosa PAO1 was able to utilize a variety of biogenic aromatic amines as a sole source of carbon or nitrogen. These amines included the phenethylamines tyramine and dopamine and the phenethanolamines octopamine and norepinephrine. Strain PAO1 could utilize synephrine as a sole source of carbon but was unable to utilize this compound or epinephrine as a sole source of nitrogen. This is possibly due to the lack of a methylamine dehydrogenase. Because of stability and ease of handling, our studies focused on tyramine and octopamine catabolism. Initial catabolism of each compound resulted in the production of the same compound, 4HPAL, although different enzymes catalyzed their respective reactions. Initial catabolism of the phenethylamines tyramine and dopamine was mediated by a membrane-bound aromatic amine dehydrogenase, which we designated TYNDH. The reaction was oxygen independent, and the enzyme could reduce the dye DCIP in the presence of tyramine (or dopamine, not shown). Although the enzyme was active only against the phenethylamines, growth of strain PAO1 with octopamine (or norepinephrine, not shown) as well as tyramine (or dopamine, not shown) resulted in the induction of similar levels of enzyme activity. This suggests either that the inducer of TYNDH is a common metabolite(s) of phenethylamine and phenethanolamine catabolism or that the specificity of induction is broad.

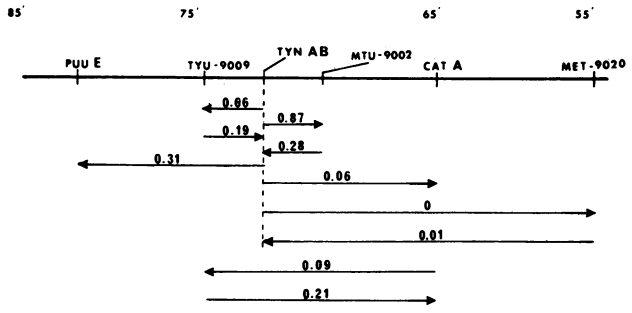

FIG. 2. Conjugational linkage map of the late region of the *P. aeruginosa* PAO1 chromosome. Numbers represent frequencies of coconjugal transfer of two markers with the chromosome-mobilizing plasmid R68.45, and arrowheads point to the unselected markers. The data represent the composite of several independent experiments in which, for each number, at least 400 to 600 transconjugants were scored. The locations of the *puuE*, *tyu-9009*, *mtu-9002*, *catA1*, and *met-9020* mutations have been determined previously (16, 17, 28).

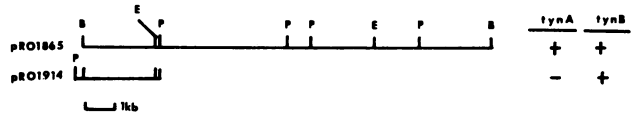

FIG. 3. Restriction endonuclease map of PAO1 chromosomal DNA fragments which complement *tyn* mutations. The *Bam*HI fragment in pRO1865 was inserted into the unique *Bam*HI site of pRO1614. The *Pst*I fragment of pRO1914 was inserted into the unique *Pst*I site of pRO1727. The ability of the plasmids to complement *tynA* and *tynB* mutations is indicated to the right. Abbreviations: B, *Bam*HI; E, *Eco*RI; P, *Pst*I.

The TYNDH of strain PAO1 resembles an aromatic primary amine dehydrogenase isolated from an unidentified *Pseudomonas* sp. able to grow on phenethylamine (19). This enzyme was active primarily against aromatic amines but is different from the TYNDH reported here. *P. aeruginosa* PAO1 cannot utilize phenethylamine as a carbon or nitrogen source, and unlike the previously reported enzyme, the addition of phenazine methosulfate to reaction mixtures with the PAO1 enzyme resulted in a significant loss of activity. In addition, the aromatic amine dehydrogenase appears to be a soluble enzyme, while the TYNDH of strain PAO1 was membrane bound. A purified soluble amine dehydrogenase from *P. putida* NP active against aliphatic and aromatic amines showed low activity against tyramine (9), but the organism was unable to utilize tyramine as a sole source of carbon (8). All previous reports on the bacterial catabolism of tyramine have shown that the initial step is mediated by a monoamine (tyramine) oxidase (21, 23, 25, 34, 35). Although the above amine dehydrogenases show activity against tyramine, this is the first report of an aromatic amine dehydrogenase mediating the initial catabolism of tyramine and dopamine.

There have been few reports on the bacterial catabolism of phenethanolamines to date. The most extensively studied reactions involve the catabolism of synephrine by *Arthrobacter synephrinum* (1, 20, 32). We have tentatively designated the first enzyme in the catabolism of phenethanolamines octopamine hydrolyase (OCAH). The

conversion of octopamine to 4HPAL appears to be similar to a novel reaction in *Arthrobacter synephrinum* (32). With both the *Arthrobacter synephrinum* catabolism of synephrine and catabolism of octopamine by *P. aeruginosa*, the product of the oxygen-independent reactions was 4HPAL; phenethylamines were not substrates. The mechanism of action of the *Arthrobacter synephrinum* enzyme is not known, but the authors have suggested a possible mechanism involving the dehydration of synephrine to form an enamine intermediate. After hydration the products 4HPAL and methylamine are formed. A similar enzyme is proposed to initiate phenethanolamine catabolism in strain PAO1. Unlike TYNDH, OCAH (measured in a combined assay with 4HPALDH) was soluble and only induced by growth with phenethanolamines.

Catabolism of 4HPAL was mediated by a soluble, NAD-dependent 4HPALDH. The product of the reaction is 4HPAA. No conversion of 4HPAL to 4HPAA was seen with the membrane fraction. Therefore, 4HPAL derived from the action of the membrane-bound TYNDH against phenethylamines is probably further metabolized by a soluble dehydrogenase. It is not known whether it is the same enzyme as that which is induced by growth with phenethanolamines. Despite repeated attempts, no mutants deficient in 4HPALDH were isolated.

Two separate classes of mutants unable to utilize tyramine or dopamine as a sole source of carbon or nitrogen were isolated. These mutants grew at wild-type rates on the phenethanolamines (octopamine and norepinephrine) and also on 4HPAA and HPA. Enzyme analysis showed that the membrane fractions of each mutant were deficient in TYNDH. As expected from the growth data, both mutant strains synthesized wild-type levels of the combined OCAH-4HPALDH activities. The two separate classes are represented by strains PAO1.299 (*tynA1*) and PAO1.222 (*tynB1*). Plasmid pRO1914 complemented the *tynB1* mutation but not *tynA1*, suggesting that the mutations are probably in separate genes.

The *tynB1* mutation was tightly linked to markers in the 70 to 75 min region of the PAO1 chromosome, analyzed with the chromosome-mobilizing conjugative plasmid R68.45. The relative frequencies of conjugational linkage of this marker to those previously mapped in the 70 to 75 min region (17) indicate that the *tynB1* mutation lies between the *mtu-9002* and *tyu-9009* mutations. Due to the tendency of PAO1.299 (*tynA1*) cells to clump, conjugal mapping experiments were difficult to perform with this strain. However, a single 13.6-kb *Bam*HI DNA fragment in pRO1865 complemented both *tynA1* and *tynB1*, locating both mutations in the same chromosomal region.

There was an asymmetry to the mapping data (Fig. 2) in crosses in which *tynB1* was the unselected marker; conjugational linkage frequencies were much lower than when *tyu-9009* or *mtu-9002* were the unselected markers. We have no explanation for this but note that a similar asymmetry, albeit in an opposite direction, has been reported previously for the linkage of *ser* to *gpu*, *aquA*, and *aquB* at min 30 of the PAO1 chromosomal map (14). The latter genes encode enzymes which release ammonia from 3-guanidinopropionate, agmatine, and *N*-carbamoylputrescine, respectively.

The *tynA1* and *tynB1* mutations were linked and mapped in a region of the PAO1 chromosome which contains many other catabolic markers (17). Nevertheless, other mutations causing a tyramine-negative phenotype mapped in other chromosomal regions (Cuskey and Olsen, manuscript in preparation), supporting the idea that the supraoperonic clustering of catabolic loci seen in *P. putida* (33) is not evident in *P. aeruginosa* (12).

Several observations suggest that an alternate catabolic pathway is used by strain PAO1 when octopamine is present as the sole source of carbon and nitrogen. When wild-type cells are grown in Burk's nitrogen-free salts medium with octopamine as the sole source of carbon and nitrogen, no combined OCAH-4HPALDH activities were seen in soluble cell extracts (Table 2), and the HPA *meta* ring fission enzyme induced by growth on tyramine and octopamine (the latter with added ammonia) was also not present (Cuskey and Olsen, manuscript in preparation). Mutants deficient in this ring fission enzyme show wild-type growth with octopamine as the sole source of carbon and nitrogen, but do not utilize octopamine as a sole source of carbon with added ammonia (data not shown). Additionally, mutants deficient in the catechol, protocatechuate, homogentisate, and gentisate ring cleavage enzymes known to exist in strain PAO1 also grew at wild-type rates with octopamine as the sole source of carbon and nitrogen (data not shown). These data suggest that an alternate pathway, different from those known to exist in strain PAO1, is responsible for catabolism of octopamine when it serves as the sole source of carbon and nitrogen. (This is not true, however, of tyramine, which is degraded via HPA under all growth conditions.) We are presently investigating this alternate pathway, which should yield novel information on the metabolic diversity of strain PAO1 and also on nitrogen regulation of phenethanolamine catabolism in this organism.

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