THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 22, Issue of November 25, pp. 13550-13556, 1982
Printed in U.S.A.

A Multispecific Quintet of Aromatic Aminotransferases That Overlap Different Biochemical Pathways in *Pseudomonas aeruginosa**

(Received for publication, May 28, 1982)

Robert J. Whitaker, Charles G. Gaines, and Roy A. Jensen

From the Department of Biological Sciences, State University of New York at Binghamton, Binghamton, New York 13901

Pseudomonas aeruginosa **possesses dual enzymatic sequences to both L-phenylalanine and L-tyrosine, a biosynthetic arrangement further complicated by the presence of five aromatic aminotransferases. Each aminotransferase is capable of transamination *in vitro* with any of the three keto acid intermediates in the aromatic pathway (phenylpyruvate, 4-hydroxyphenylpyruvate, or prephenate). The fractional contribution of these aminotransferases to particular transamination reactions *in vivo* can best be approached through the systematic and sequential elimination of individual aminotransferase activities by mutation. A program of sequential mutagenesis has produced two aminotransferase-deficient mutations. The first mutation imposed a phenotype of bradytrophy for L-phenylalanine (doubling time of 2.4 h in minimal salts/glucose medium compared to a 1.0-h doubling time for wild type). This mutant completely lacked an enzyme denoted aminotransferase AT-2. A genetic background of aminotransferase AT-2 deficiency was used to select for a second mutation which produced a phenotype of multiple auxotrophy for L-phenylalanine, L-aspartate, and L-glutamate. The double mutant completely lacked activity for aromatic aminotransferase AT-1 in addition to the missing aminotransferase AT-2. Enzymes AT-1 (M_r = 64,000) and AT-2 (M_r = 50,000) were readily separated from one another by gel filtration and were individually characterized for pH optima, freeze-thaw stability, heat lability, and molecular weight. The phenotypic and enzymological characterizations of the aminotransferase mutants strongly support the primary *in vivo* role of enzyme AT-2 in L-phenylalanine and L-tyrosine biosynthesis, while enzyme AT-1 must primarily be engaged in L-aspartate and L-glutamate synthesis. The substrate specificities and possible *in vivo* functions for AT-3, AT-4, and AT-5 are also considered.**

While some aminotransferases may be highly specialized for a particular transamination reaction, this group of enzymes as a whole is perhaps the prime example of substrate overlap exhibited by a family of enzyme proteins.[1] This breadth of substrate specificity considerably complicates the ease of establishing gene-enzyme relationships owing to the lack of mutant strains. Single aminotransferase mutations are sometimes phenotypically silent, as their absence may be masked in the presence of other aminotransferase species of overlapping catalytic potential. Thus, in *Bacillus subtilis* the *hisH* aminotransferase (which is obligatory for L-histidine biosynthesis) can also function as an aromatic aminotransferase in the biosynthesis of L-phenylalanine and L-tyrosine. Indeed, the sole presence of either *hisH* or of the aromatic aminotransferase (*aroJ*) is adequate for L-phenylalanine and L-tyrosine biosynthesis (2).

The fractional contribution of such broad spectrum aminotransferase families to a particular transamination *in vivo* can be approached through the sequential elimination of individual aminotransferases by mutation. Any strategy of sequential mutagenesis is dependent upon the fortuitous existence of a selectable phenotype, allowing recognition of a first step mutation. The elimination of an aminotransferase that functionally overlaps other biochemical pathways may create an increased dependence of these pathways on the remaining aminotransferase species. Therefore, the introduction of a second aminotransferase deficiency in a genetic background stressed by the absence of one aminotransferase species may yield a selectable phenotype that might have otherwise been suppressed in the presence of the first aminotransferase. Mutations that are accumulated in sequence can then be separated or sorted into different combinations by using appropriate genetic techniques, thereby allowing the characterization of individual mutants whose phenotypic alterations may be very subtle.

An impressive application of sequential mutagenesis has been reported in *Escherichia coli* K-12 (3), where any one of three aminotransferases (coded by *ilvE, tyrB,* or *aspC*) alone is adequate to transaminate phenylpyruvate in L-phenylalanine biosynthesis. Indeed, the degree of functional overlap is such that the joint elimination of the aminotransferases specified by *tyrB* and *aspC* is masked in the presence of wild type *ilvE*. Beginning with an *ilvE*-deficient parental background (requires isoleucine), Gelfand and Steinberg (3) isolated an *ilvE tyrB* double mutant (requires isoleucine, valine, leucine, and tyrosine), from which an *ilvE tyrB aspC* triple mutant (requires isoleucine, valine, leucine, tyrosine, phenylalanine, and aspartate) was obtained. Phenotypic analysis of these mutations assembled in various combinations by recombination defined the *in vivo* function of all three aminotransferases.

In *Pseudomonas aeruginosa* tightly blocked auxotrophs for phenylalanine or tyrosine are not obtained as the result of single mutations. The enzymological basis for this "reluctant auxotrophy" (4) has been attributed to the joint presence of dual biosynthetic pathways to L-phenylalanine and to L-tyrosine (5). This arrangement,[2] shown in Fig. 1, is presumed to

* These investigations were supported by Grant AM-19447 from the United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] See Ref. 1 (pp. 237-241) for an historical development of this topic, beginning with the findings of Rudman and Meister (23), who first demonstrated the overlapping specificities of microbial aminotransferases.

[2] L-Arogenate was first named pretyrosine. With the eventual appreciation that this amino acid may also be a precursor for L-phenylalanine biosynthesis (see Ref. 22 for a review), the more appropriate name of L-arogenate was adopted. The Greek roots of aro-genate (*gen*, giving rise to; *aro*, aromatic compound) imply the broad precursor roles for L-arogenate that are now coming to light.

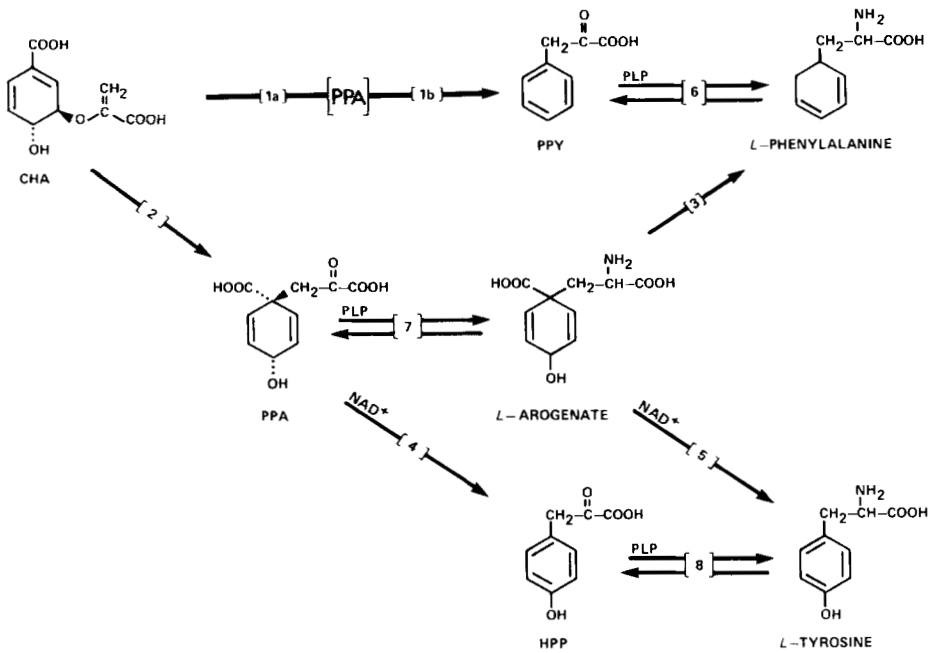

FIG. 1. **Dual biosynthetic routes of L-phenylalanine and L-tyrosine** in *P. aeruginosa*. Enzymes: *1a*, chorismate (*CHA*) mutase; *1b*, prephenate (*PPA*) dehydratase (enzymes *1a* and *1b* form a bifunctional protein (5) in which PPA is an enzyme-bound intermediate); *2*, chorismate mutase II; *3*, arogenate dehydratase; *4*, prephenate dehydrogenase; *5*, arogenate dehydrogenase; *6*, phenylpyruvate (*PPY*) aminotransferase; *7*, prephenate aminotransferase; *8*, 4-hydroxyphenylpyruvate (*HPP*) aminotransferase. All five aminotransferase species can catalyze reactions *6*, *7*, and *8*. *NAD*$^+$, nicotinamide adenine dinucleotide; *PLP*, pyridoxal 5'-phosphate.

preclude the isolation of stringent phenylalanine or tyrosine auxotrophs, since a mutant interruption of one branchlet sequence would be masked by the availability of an intact alternative branchlet to the relevant end product. A further seeming complication is the finding that *P. aeruginosa* possesses a larger number of aminotransferase enzymes (five) capable of function as aromatic aminotransferases *in vitro* than is usually found. For example, *B. subtilis* (2), *Corynebacterium glutamicum* (6), and *Brevibacterium flavum* (6) each have two enzyme species, while *E. coli* exhibits three species (3). Nevertheless, the only significant impact upon nutritional phenotype thus far known caused by loss of any postprephenate enzyme in *P. aeruginosa* is a lack of aromatic aminotransferase AT-2, which leads to phenylalanine bradytrophy (4). This mutant is shown to provide a suitable genetic background for the beginning of a systematic elimination of aromatic pathway enzymes through further mutations.

MATERIALS AND METHODS

Microbiological Aspects

The PAO strain of *P. aeruginosa* (wild type) was originally obtained from B. W. Holloway, University of Melbourne (7). Isolation of the leaky L-phenylalanine auxotroph NP-72 was previously described (4). The double mutant RW8-27 was derived from NP-72 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis using the procedures of Calhoun and Feary (8). Unless otherwise specified, cultures were grown at 37 °C in a minimal salts medium (9) containing either 0.5% glucose or fructose. Solid medium for plating contained 1.5% (w/v) Difco agar. Any nutritional supplements specified were added to a final concentration of 50 μg/ml.

The doubling times of wild type, NP-72, and RW8-27 were determined by monitoring cell turbidity using a Klett-Summerson colorimeter. A 54 green filter was used to avoid erroneous readings otherwise caused by pyocyanine pigment. Overnight cultures grown in minimal salts/glucose medium (wild type) supplemented with 50 μg/ml of L-phenylalanine (NP-72) or supplemented with 50 μg/ml of L-phenylalanine, L-aspartate, and L-glutamate (RW8-27) were harvested by centrifugation, resuspended in 5 ml of sterile medium, and used to inoculate 10 ml of fresh medium containing the amino acid supplements indicated in Table I. Cultures were grown in 125-ml side arm flasks with vigorous shaking at 37 °C. The starting turbidities for growth curves ranged from 20–30 Klett units.

When growing cultures for extract preparation, overnight cultures (200 ml) were used to inoculate 20 liters of minimal salts/glucose medium containing any amino acid supplements indicated at 50 μg/ml. Cells were incubated at 37 °C with continuous aeration and harvested at late exponential phase of growth by centrifugation. Cell pellets were washed with 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM $MgSO_4$ and 10% glycerol and frozen at −80 °C until needed.

TABLE I

Growth responses of aminotransferase mutants

Strain[a]	Nutritional additions[b]	Doubling time
		h
Wild type	Minimal salts/glucose	1.0
Single mutant (NP-72)	Minimal salts/glucose	2.4
	+ L-Phenylalanine	1.0
	+ L-Tyrosine	1.6
	+ L-Aspartate	2.4
	+ L-Glutamate	2.5
Double mutant (RW8-27)	Minimal salts/glucose	
	+ L-Phenylalanine	4.5
	+ L-Tyrosine	9.0
	+ L-Aspartate	4.6
	+ L-Glutamate	4.2
	+ L-Aspartate, L-glutamate	2.9
	+ L-Phenylalanine, L-aspartate	2.0
	+ L-Tyrosine, L-aspartate	3.0
	+ L-Phenylalanine, L-glutamate	2.1
	+ L-Phenylalanine, L-aspartate, L-glutamate	1.1
	+ L-Phenylalanine, L-tyrosine, L-aspartate, L-glutamate	1.0

[a] NP-72 is a phenylalanine bradytroph; RW8-27 is leaky on L-phenylalanine, L-glutamate, L-aspartate, or L-tyrosine but fails to grow at all on minimal salts/glucose medium.

[b] Supplements to a minimal salts/glucose medium were present at a final concentration of 50 μg/ml.

Crude Extract Preparation

Cell pellets were thawed and washed in 3 volumes of 50 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM dithiothreitol and 0.05 mM pyridoxal 5'-phosphate. Crude extracts were prepared by sonication using a Lab-Line Ultra-tip sonicator. After sonication, the extract was centrifuged at 150,000 × *g* to remove cell debris. The resulting supernatant was then passed through a Sephadex G-25 column (1.5 × 20 cm) equilibrated in starting buffer to remove small molecules that might complicate enzyme assays.

Aminotransferase Assays

In some experiments, particularly when monitoring elution profiles from chromatographic columns, a spectrophotometric assay for phenylpyruvate at 320 nm was used. Reaction mixtures contained 10 mM L-phenylalanine, 2.5 mM α-ketoglutarate, 12.5 μM pyridoxal 5′-phosphate, 50 mM potassium phosphate buffer (pH 7.0), and enzyme. All reaction mixtures were incubated at 37 °C for 20 min and then terminated by addition of 800 μl of 2.5 N NaOH. An extinction coefficient of 17,500 (10) was used to calculate the concentration of phenylpyruvate formed.

In experiments requiring more precision, a procedure employing radiolabeled amino acids was used. A method previously described for the assay of α-ketoglutarate/tyrosine aminotransferase (11) was used with some modification (12). This method allowed a broader appraisal of aminotransferase reactivities with a range of amino donor/keto acceptor combinations. As this assay depends upon the extraction of radioactive keto acid formed into ethylacetate:toluene (4:1), the differing extraction efficiencies of the various keto acids must be considered in calculating specific activities. The extraction efficiencies are: phenylpyruvate, 98%; 4-hydroxyphenylpyruvate, 100%; α-ketoglutarate, 21%; oxaloacetate, 19%; and α-ketoisocaproate, 70%. Reaction mixtures (200 μl) contained 50 mM potassium phosphate buffer, 25 μg of bovine serum albumin, 25 μM pyridoxal 5′-phosphate, 2.5 mM keto acid, 2.5 mM L-[^{14}C]aminoacid, and enzyme. The L-[^{14}C]aminoacids tested were: phenylalanine, tyrosine, glutamate, aspartate, and leucine, while the keto acids tested were: phenylpyruvate, 4-hydroxyphenylpyruvate, prephenate, α-ketoglutarate, and oxaloacetate. After incubation at 37 °C for 20 min, the reaction mixture was acidified by addition of 0.3 ml of 1.0 N HCl. The radioactive keto acid product was then extracted in 1.0 ml of ethylacetate:toluene (4:1). Duplicate 200-μl samples of the organic phase were then transferred separately to 10 ml of Liquifluor and counted in a Packard scintillation counter.

Other Analytical Techniques

Chorismate mutase, prephenate dehydratase, arogenate dehydratase, prephenate dehydrogenase, arogenate dehydrogenase, and 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase were assayed as previously described (13–15).

Protein was measured by the method of Lowry *et al.* (16).

Enzyme Fractionation Procedures

DEAE-cellulose Chromatography—Crude extracts of wild type, NP-72, and RW8-27 were applied to a DEAE-cellulose column (Whatman DE52) (5 × 30 cm) previously equilibrated in 50 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM dithiothreitol and 0.05 mM pyridoxal 5′-phosphate. Protein was washed on the column with 1 liter of starting buffer. Protein was eluted by a linear salt gradient (0 to 0.5 M KCl). Fractions of 6 ml were collected at 4 °C. Elution of aminotransferase activity was measured with the L-phenylalanine:α-ketoglutarate assay, and peak fractions were pooled and concentrated by Amicon PM-10 filtration.

Hydroxylapatite Chromatography—Fractions eluted within the peak denoted DE-II were applied to a Sephadex G-25 column (1.5 × 20 cm) equilibrated in 10 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM dithiothreitol and 0.05 mM pyridoxal 5′-phosphate in order to reduce the phosphate concentration. The protein recovered was then loaded on a hydroxylapatite column (1.5 × 10 cm) and washed with 100 ml of 10 mM potassium phosphate buffer (pH 7.0) plus 1.0 mM dithiothreitol and 0.05 mM pyridoxal 5′-phosphate. A linear phosphate gradient (10 to 200 mM) was used to elute the protein. Fractions of 2.2 ml were collected at 4 °C. Activities were measured by the L-phenylalanine:α-ketoglutarate assay.

Ultrogel AcA34 Chromatography—Fractions eluted from DE52 within the peak denoted DE-I were applied to Sephadex G-25 to reduce the buffer concentration and then loaded on an Ultrogel AcA34 gel filtration column (2.5 × 60 cm) equilibrated in 10 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM dithiothreitol and 0.5 mM pyridoxal 5′-phosphate. Fractions of 3 ml were collected and assayed for aminotransferase activity by the L-phenylalanine:α-ketoglutarate spectrophotometric assay. The gel column was calibrated with the following molecular weight standards: catalase (210,000), aldolase (158,000), bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen A (25,000), and chymotrypsin (22,500). The void volume was determined with blue dextran.

Biochemicals and Chemicals

Amino acids, keto acids, and Sephadex G-25 were obtained from Sigma. DEAE-cellulose (DE52) was obtained from Whatman and hydroxylapatite was acquired from Bio-Rad. All L-^{14}C-labeled amino acids and Liquifluor were obtained from New England Nuclear. Chorismate was prepared from *Klebsiella pneumoniae* 62-1 (17); barium prephenate (99% pure) was prepared from *Salmonella typhimurium* (18) and converted to the potassium salt with excess K_2SO_4 prior to use; L-arogenate (>90% pure) was prepared from *Neurospora crassa* (19).

RESULTS

Strategies for Isolation of Mutants Blocked in L-Phenylalanine Biosynthesis—Routine mutagenesis of *P. aeruginosa* has not produced phenylalanine or tyrosine auxotrophs under conditions where a good yield of other auxotrophs was obtained. This reluctant auxotrophy has been explained as the consequence of the unusual dual pathway branchlets that lead to both L-phenylalanine and to L-tyrosine in this organism (5). One promising approach to offset the latter complication in isolating mutants is to depress the flow of carbon into the aromatic pathway, an objective which can be accomplished simply by growth on fructose as sole source of carbon (9). The transport of fructose evidently stresses the intracellular supply of phosphoenolpyruvate, since a P-enolpyruvate-dependent phosphotransferase system of transport is used (20). Two molecules of P-enolpyruvate (*i.e.* enzymes 1 and 6 of the common pathway) are required to synthesize each molecule of the three aromatic amino acids. The use of fructose medium did indeed lead to the recovery of a leaky phenylalanine auxotroph (4). This bradytrophic strain (NP-72) proved to be deficient in aromatic aminotransferase activity. We reasoned that an absolute requirement for L-phenylalanine might require more than a single mutation. Hence, mutant NP-72 was used as the parental background in a second mutagenesis treatment in which an absolute requirement for L-phenylalanine was sought in the presence of other amino acids. This yielded the double mutant, RW8-27, which requires phenylalanine, aspartate, and glutamate to sustain wild type growth rates.

The nutritional requirements of mutants NP-72 and RW8-27 are illustrated by the growth rates shown in Table I. Mutant NP-72 will only grow at the wild type rate in the presence of L-phenylalanine. The presence of L-tyrosine alone stimulates the growth rate significantly but does not restore the wild type growth rate. The double mutant, RW8-27, does not grow at all in minimal salts/glucose medium, but does, however, exhibit leaky growth in the presence of either L-phenylalanine, L-glutamate, L-aspartate, or L-tyrosine alone.

Identification of Gene-product Deficiencies in Mutant Strains—Crude extracts of the mutant strains and wild type were prepared and chromatographed on DEAE-cellulose. Activities of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase, chorismate mutase, prephenate dehydratase, prephenate dehydrogenase, arogenate dehydratase, and arogenate dehydrogenase were all found to be present and similar in each extract with respect to specific activity and chromatographic position (data not shown). The mutants did differ from wild type in the profiles found for aromatic aminotransferase activities as illustrated in Figs. 2 and 3. In wild type, DEAE-cellulose chromatography separated two fractions[3] of

[3] Aminotransferase species AT-2, AT-3, AT-4, and AT-5 correspond to the four previously described (4, 5) aminotransferases having the nomenclature DE-I, HA-I, HA-II, and HA-III. The very labile species AT-1 was not detected previously. In the DEAE-cellulose fractionation, two aminotransferase bands were separated (band DE-I containing a mixture of species AT-1 and AT-2 and band DE-II containing a mixture of species AT-3, AT-4, and AT-5).

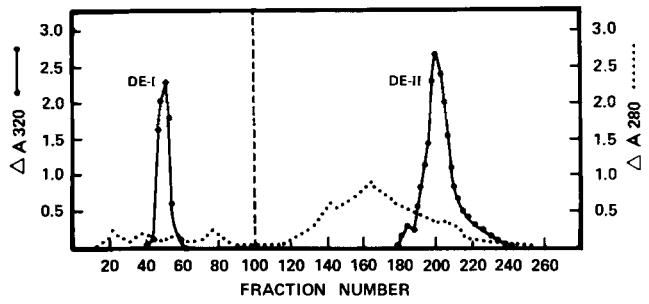

FIG. 2. **Partial resolution of aromatic aminotransferases by DEAE-cellulose (DE52) chromatography.** A crude extract prepared from a wild type cell population grown in minimal salts/glucose medium (specific activity by phenylalanine/α-ketoglutarate assay = 67.7 nmol/min/mg) was applied to a DE52 column (5 × 30 cm). The activity band eluted in the wash fractions was denoted DE-I (specific activity = 100 nmol/min/mg), and the activity band eluted in the gradient was denoted DE-II (specific activity = 119.8 nmol/min/mg). Similar results were obtained when extracts from mutants NP-72 and RW8-27 were chromatographed on DE52, except that (*i*) the amount of the DE-I band in mutant NP-72 was much reduced, and (*ii*) the DE-I band was absent altogether in mutant RW8-27.

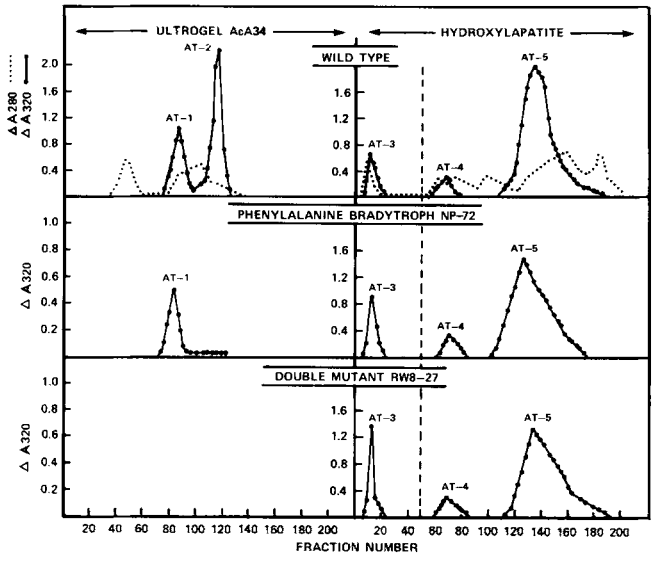

FIG. 3. **Comparison of aminotransferase profiles of wild type with [AT-2]-deficient mutant NP-72 and the double mutant RW8-27 lacking both [AT-1] and [AT-2] species of aminotransferase.** The distribution of protein as estimated by A_{280} and shown only in the *top* was essentially identical in the profiles given in the other panels. *Top*, the DE-I aminotransferase band (fractions 44–62) shown in Fig. 2 were pooled, concentrated by Amicon PM-10 filtration, and applied to an Ultrogel AcA34 column (see under "Materials and Methods"). Two aminotransferase species, denoted AT-1 and AT-2, were eluted as shown on the left. Activity band DE-II recovered as shown in Fig. 2 was concentrated and applied to an hydroxylapatite column (see under "Materials and Methods"), yielding three separate bands of aminotransferase activity denoted AT-3, AT-4, and AT-5. The specific activities (nanomoles/min/mg) of the five aminotransferases were: AT-1, 120; AT-2, 179; AT-3, 45; AT-4, 13; and AT-5, 320 (using the substrate combination of phenylalanine and α-ketoglutarate). *Middle*, a crude extract of minimal salts/glucose-grown cells from mutant NP-72 was chromatographed over DE52 as specified in Fig. 2. Gel filtration and hydroxylapatite fractionations were carried out as above. The specific activities of the four aminotransferase bands recovered were: AT-1, 108; AT-3, 55; AT-4, 17; and AT-5, 358. *Bottom*, double mutant RW8-27 was grown in minimal salts/glucose medium containing 50 μg/ml each of L-phenylalanine, L-aspartate, and L-glutamate. Aminotransferase band DE-II was recovered from a DE52 chromatography step as in Fig. 2, concentrated, and applied to a hydroxylapatite column as described above. The specific activities of the three aminotransferase bands recovered were: AT-3, 209; AT-4, 6.4; and AT-5, 292.

aromatic aminotransferase. The leftward peak, denoted as peak DE-I, migrated through the column without retardation and was recovered in the wash eluate. The remaining activity eluted in the gradient as a complex mixture of overlapping peaks, denoted as peak DE-II. In mutant NP-72 the magnitude of peak DE-I was clearly diminished (to a peak-tube absorbance of 0.80 at 320 nm), while mutant RW8-27 lacked peak DE-I altogether.

Peak DE-II fractions were combined, concentrated by Amicon PM-10 filtration, and passed through a hydroxylapatite column. In wild type and both mutant strains, this cleanly resolved three aminotransferase enzymes denoted AT-3, AT-4, and AT-5 (rightward panels of Fig. 3). The presence of L-phenylalanine during growth characteristically results in elevation of species AT-3 (about 4- to 5-fold) and repression of species AT-4 (about 2- to 3-fold). These regulatory effects, previously observed (4), can be seen comparing specific activities given in the legend of Fig. 3 where RW8-72 was grown in the presence of L-phenylalanine, unlike the other two strains.

The DE-I fractions from wild type were combined, concentrated by Amicon PM-10 filtration, and passed through a gel filtration column (Fig. 3, *top left*). This resolved two aminotransferases; the larger (M_r = 64,000) denoted aminotransferase AT-1 and the smaller (M_r = 50,000) denoted aminotransferase AT-2. Mutant NP-72 differed from wild type in the absence of aminotransferase AT-2, while mutant RW8-27 lacked both aminotransferases AT-1 and AT-2. These data are consistent with the diminished magnitude and the absence of peak DE-I seen for mutants NP-72 and RW8-27, respectively, in Fig. 2.

Overlapping Aminotransferase Substrate Specificities—

TABLE II

Substrate specificities of the aromatic aminotransferases

Aromatic aminotransferases were partially purified as described in the legends of Figs. 2 and 3.

Keto acid substrate	^{14}C-labeled L-amino acid substrate	Specific activity				
		[AT-1]	[AT-2]	[AT-3]	[AT-4]	[AT-5]
		nmol/mg/min				
Phenylpyruvate	Phenylalanine	21.7	14.6	0.8	0.1	5.9
	Tyrosine	45.1	14.7	0.5	0.2	0.4
	Glutamate	216.4	25.7	38.3	4.9	4.4
	Aspartate	52.1	18.5	2.9	0.6	0.0
	Leucine	1.0	1.9	2.5	3.0	43.5
4-Hydroxyphenyl-pyruvate	Phenylalanine	20.7	15.1	1.4	0.1	6.3
	Tyrosine	49.0	13.9	0.6	0.3	0.7
	Glutamate	238.0	24.5	12.8	2.1	4.0
	Aspartate	62.8	25.6	0.1	0.4	0.0
	Leucine	0.8	2.0	0.5	2.1	31.6
Prephenate	Phenylalanine	13.0	11.2	0.9	0.1	10.7
	Tyrosine	23.6	13.0	3.2	1.1	2.9
	Glutamate	42.3	20.9	28.8	5.1	15.8
	Aspartate	16.6	20.3	3.8	0.9	0.0
	Leucine	0.9	1.8	2.1	3.2	7.3
α-Ketoglutarate	Phenylalanine	31.0	9.4	0.9	0.3	19.5
	Tyrosine	53.5	8.4	4.0	1.6	6.0
	Glutamate	408.7	78.0	69.2	9.8	45.9
	Aspartate	252.0	46.5	2.5	1.4	0.1
	Leucine	0.9	1.1	4.9	2.5	78.3
Oxaloacetate	Phenylalanine	12.0	9.4	0.4	0.1	2.2
	Tyrosine	14.0	9.9	2.2	0.8	1.6
	Glutamate	33.0	12.3	0.0	0.0	5.3
	Aspartate	16.2	11.5	0.0	0.3	0.2
	Leucine	0.7	2.3	0.0	0.0	5.1
α-Ketoisocaproate	Phenylalanine	0.0	0.0	0.3	0.2	6.3
	Tyrosine	0.1	0.0	0.1	0.0	1.8
	Glutamate	0.0	0.2	0.1	0.0	0.3
	Aspartate	0.0	0.3	0.0	0.0	10.4
	Leucine	0.0	0.3	0.3	0.3	44.9

The specific activities of all five aminotransferases with various amino donor/keto acceptor combinations are listed in Table II. The best amino donor substrate for enzymes AT-1, AT-2, AT-3, and AT-4 was L-glutamate, while enzyme AT-5 ultilized L-leucine best. Aminotransferase AT-1 was the most active enzyme with all three keto acids of aromatic biosynthesis (*i.e.* prephenate, phenylpyruvate, and 4-hydroxyphenylpyruvate), although all five aminotransferases demonstrated activity with the three keto intermediates of L-phenylalanine and L-tyrosine biosynthesis. Enzyme AT-2 roughly parallels the substrate specificities of enzyme AT-1, but is generally much less active. Owing to its inducibility by L-phenylalanine or by L-tyrosine, aminotransferase AT-3 is thought to be a catabolic enzyme (4). However, its anabolic potential has been demonstrated (4) in suppressor mutants where constitutive expression of enzyme AT-3 suppresses the NP-72 defect (lack of AT-2). Utilizing L-glutamate as an amino donor, enzyme AT-3 demonstrated reasonable enzyme activity in the biosynthetic direction using phenylpyruvate, 4-hydroxyphenylpyruvate, or prephenate as keto substrates. Although enzyme AT-4 exhibited comparatively low activity, repression of this enzyme by L-phenylalanine or L-tyrosine (4) implicates a biosynthetic role in L-phenylalanine and L-tyrosine synthesis. It is interesting to note that AT-4 utilizes prephenate as well or better than any other keto acceptor, a result not observed with the other four aminotransferases. Although enzyme AT-5 appears to be generally reactive with all the keto substrates tested, it was much more reactive with α-ketoisocaproate than was any of the other four aminotransferase species. It seems likely that the primary *in vivo* role of enzyme AT-5 is in branched chain amino acid metabolism.

Further Characterization of Aminotransferases AT-1 and AT-2—Aside from the molecular weight distinction, enzymes AT-1 and AT-2 differ in a number of physical characteristics. Enzyme AT-2 showed similar pH profiles (Fig. 4, *right*) with optima at pH 7.0 when L-phenylalanine, L-tyrosine, L-aspartate, or L-glutamate were used in combination with α-ketoglutarate. In contrast, enzyme AT-1 (Fig. 4, *left*) exhibited pH optima that varied strikingly with different amino donor reactants: pH 8.4 with either L-phenylalanine or L-tyrosine, pH 7.6 with L-aspartate, and pH 6.2 with L-glutamate.

Enzyme AT-1 was unstable to freeze-thaw treatment as shown in Fig. 5. With a single freeze-thaw cycle enzyme AT-1 lost about 50% of its original activity (*left*), while enzyme AT-2 remained stable (*middle*). Additional freeze-thaw treatments completely inactivated enzyme AT-1, while enzyme AT-2 remained stable. Enzyme AT-1 was also distinctly more labile to heat treatment (*right*). Both aminotransferases were inactivated at 60 °C, but enzyme AT-1 lost activity much more precipitously than did enzyme AT-2.

These latter differential characteristics of aminotransferases AT-1 and AT-2 were used to confirm the identity of peak DE-I (Fig. 2) in mutant NP-72 as enzyme AT-1, resolved (Fig. 3) from peak DE-I of wild type. Crude extracts of NP-72, grown on minimal salts/glucose medium, were prepared and chromatographed on DEAE-cellulose exactly as with wild type (see Fig. 2, *left*). The wash eluate of activity corresponding to peak DE-I was pooled and concentrated as before and chromatographed on Ultrogel AcA34 (Fig. 3, *middle*). A single peak corresponding to aminotransferase AT-1 was eluted. To confirm the identity of this activity as enzyme AT-1, a characterization of molecular weight, pH optimum, freeze-thaw stability, and heat lability were determined. The data summarized in Table III demonstrate that the DE-I peak, which in wild type is a mixture of enzymes AT-1 and AT-2, consists solely of species AT-1 in mutant NP-72.

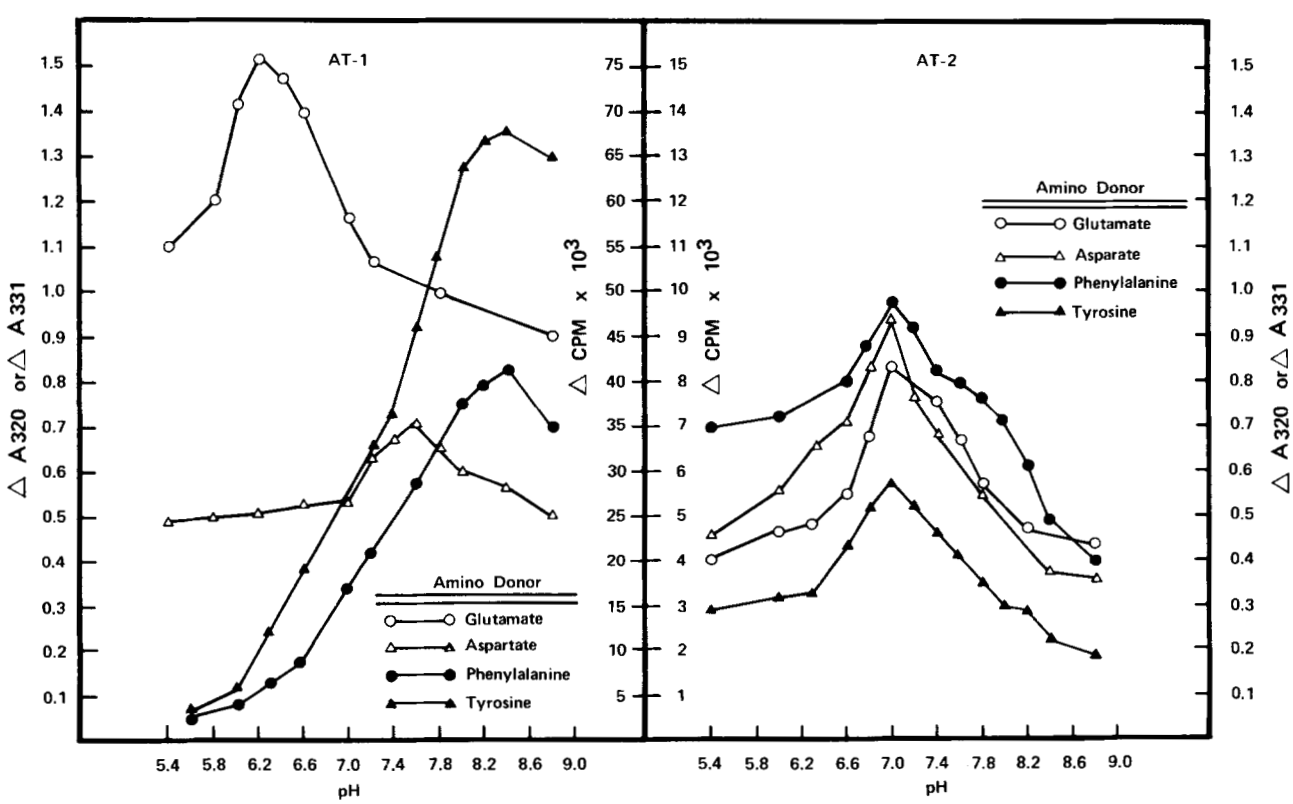

FIG. 4. **pH optima for aromatic aminotransferases AT-1 and AT-2.** Buffers at a final concentration of 0.1 M were citrate-phosphate in the pH range of 5.4–6.0, potassium phosphate in the pH range of 6.0–8.0, and Tris·HCl in the pH range of 8.0–9.0. In all assays α-ketoglutarate was keto acceptor substrate. When L-phenylalanine or L-tyrosine was the amino donor, the spectrophotometric assay of phenylpyruvate (320 nm) or 4-hydroxyphenylpyruvate (331 nm) was used. When the amino donor was L-aspartate or L-glutamate, ^{14}C-labeled amino acids were used (see under "Materials and Methods").

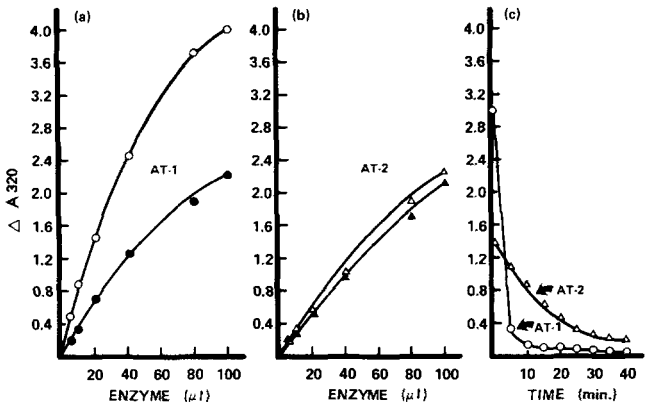

FIG. 5. **Differential stability characteristics of aminotransferase species AT-1 and AT-2.** The substrate combination of phenylalanine and α-ketoglutarate was used in all activity determinations. Partially purified preparations of enzyme AT-1 (*a*) and enzyme AT-2 (*b*) obtained as specified under Fig. 3 were frozen at −80 °C overnight and thawed at 37 °C. Enzyme velocity as a function of enzyme concentration is shown before (*open symbols*) and after (*closed symbols*) a cycle of freeze-thaw treatment. (*c*) shows the differential heat lability of the two enzymes. Samples of AT-1 and AT-2 were incubated at 60 °C, and 50-μl aliquots were withdrawn at 5-min intervals and kept over ice until all samples had been collected.

TABLE III
Identification of aminotransferase AT-1 in NP-72

Strain	Enzyme[a]	Freeze-thaw stability[b]	Heat stability[c]	pH optimum[d]	Molecular weight[e]
Wild type	AT-2	Stable	Labile ($t_{1/2}$ = 1.3)	7.0	50,000
Wild type	AT-1	Labile	Labile ($t_{1/2}$ = 2.5)	8.4	64,000
NP-72	AT-1	Labile	Labile ($t_{1/2}$ = 3.0)	8.4	64,000

[a] Samples of AT-1 and AT-2 were obtained after Ultrogel AcA34 chromatography (see Fig. 3).

[b] The NP-72 samples of AT-1 and the wild type enzymes AT-1 and AT-2 were assayed for phenylalanine:α-ketoglutarate activity both before and after freezing and thawing as described in the legend of Fig. 5.

[c] See legends of Fig. 5 for procedures. The numbers in parentheses represent the amount of time (minutes) required for a 50% loss of activity.

[d] See legend of Fig. 5 for details.

[e] Molecular weight was determined by Ultrogel AcA34 gel filtration (see under "Materials and Methods").

DISCUSSION

Dilemma of Dual Pathways for Aromatic Biosynthesis—Various pseudomonad organisms have been found to possess a divergent array of enzyme sequences from prephenate to L-phenylalanine, from prephenate to L-tyrosine, or to both end products (13–15). The most studied organism is *P. aeruginosa*, which has dual branchlets leading to both end products (Fig. 1). The fractional flow of intermediates along the competing branchlets is as yet unknown. However, it seems probable that the objective of isolating mutants auxotrophic for phenylalanine or tyrosine is thwarted as the consequence of an intact alternate flow route. Mutant phenotypes are apparently masked, since it is probable that no mutation blocks both branchlets simultaneously. This metabolic complexity is exacerbated by the finding that the three aminotransferase activities (Reactions 6 to 8 in Fig. 1) are represented by five proteins *in vivo*, each capable of carrying out all three reactions *in vitro*.

Strategies for Isolation of Deficient Mutants—If any of the nine postprephenate enzymes (*i.e.* five aminotransferases, two dehydratases, and two dehydrogenases) were essential in order to sustain the full wild type rate of growth, then the loss of that enzyme should cause leaky (bradytrophic) growth. If the flow of carbon entering aromatic biosynthesis is slowed, then the chances of creating a growth-limiting condition through loss of one of the nine enzymes would be increased. We showed that growth on fructose renders aromatic biosynthesis rate-limiting (9), undoubtedly at the level of P-enolpyruvate since a P-enolpyruvate-dependent transport system is employed for fructose transport in *P. aeruginosa* (20). Indeed, a mutant bradytrophic for L-phenylalanine (NP-72) was isolated using the approach of selection on fructose-containing medium (4). We expected this mutant to be deficient in either prephenate dehydratase or in arogenate dehydratase. Surprisingly, even though each aminotransferase step is represented by five proteins capable of catalyzing the reaction *in vitro*, mutant NP-72 proved to be deficient in aminotransferase AT-2 (4). Although Patel *et al.* (4) worked with band DE-I (which we show here to be a mixture of enzyme species AT-1 and AT-2), it seems clear that Patel *et al.* only studied species AT-2 because of freeze-thaw handling that dramatically inactivates species AT-1. Their characterization of "species DE-I" corresponds well with our characterization of species AT-2.

Does the lack of enzyme AT-2 in mutant NP-72 decrease the activity of one aminotransferase reaction *in vivo, e.g.* phenylpyruvate aminotransferase? Or does loss of enzyme AT-2 jointly diminish transamination *in vivo* of phenylpyruvate, prephenate, and 4-hydroxyphenylpyruvate? The latter seems likely since L-tyrosine alone partially restores the growth rate of NP-72 in minimal salts medium. L-Tyrosine may be less effective than L-phenylalanine simply because the sparing effect of tyrosine addition may be offset by the potent feedback inhibition of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase by L-tyrosine (21), thereby depleting substrate supply to aromatic aminotransferase reactions.

Having first limited aromatic biosynthesis physiologically through use of fructose as carbon source and genetically following isolation of NP-72, a second step of sequential mutagenesis was feasible for selection of additional enzyme deficiencies. We isolated a derivative of NP-72, mutant RW8-27, which failed to grow on minimal salts medium but which responded partially to L-phenylalanine, L-tyrosine, L-glutamate, or L-aspartate. Mutant RW8-27 requires the combination of phenylalanine, L-aspartate, and L-glutamate to achieve the wild type growth rate. It proved to lack aminotransferase AT-1 in addition to the previously missing AT-2 enzyme. Since the double mutant can grow, albeit slowly, in the absence of aromatic supplements (*e.g.* on L-aspartate or on L-glutamate), enzymes AT-3, AT-4, and AT-5 must be limited in ability to transaminate with aromatic pathway keto acids to the extent that growth rate is slowed more than 4-fold. Species AT-3 has been shown (4) to be able to compensate for a deficiency of enzyme AT-2 when levels of AT-3 are elevated following a suppressor mutation which causes the normally inducible AT-3 enzyme to be expressed constitutively.

It should now be possible to use the double aminotransferase mutant as genetic background to eliminate some or all of the remaining aminotransferases. Multiple mutants lacking all species of aminotransferase capable of utilizing aromatic pathway compounds should be capable of growth on L-arogenate in substitution for L-phenylalanine and L-tyrosine. Against this background mutants deficient in arogenate dehydratase and arogenate dehydrogenase should be selectable. Given the isolation, for example, of an arogenate dehydrogenase-deficient mutation by these means, aminotransferase activities could be restored to provide a genetic background where loss of the gene for prephenate dehydrogenase would be selectable as a tightly blocked tyrosine auxotroph. Eventually all individual mutations accumulated by sequential

TABLE IV

Summary comparison of aromatic aminotransferase properties in P. aeruginosa

The five molecular species were partially purified as in Figs. 2 and 3. Abbreviations used are PHE, L-phenylalanine; TYR, L-tyrosine; GLU, L-glutamate; ASP, L-aspartate; LEU, L-leucine; HPP, 4-hydroxyphenylpyruvate; PPY, phenylpyruvate; and PPA, prephenate.

Amino-transferase	Molecular weight	Heat stability[a]	Freeze-thaw stability[a]	pH optima[a]	Amino donor preference[b]	Keto acceptor preference[b]	Regulation by PHE or TYR	Major *in vivo* role postulated
AT-1	64,000	Very labile	Labile	PHE 8.4 TYR 8.4 GLU 6.2 ASP 7.6	GLU > ASP ≫ LEU	HPP = PPY > PPA	None	Biosynthesis: glutamate, aspartate
AT-2	50,000	Labile	Stable	PHE 7.0 TYR 7.0 GLU 7.0 ASP 7.0	GLU = ASP > LEU	HPP = PPY = PPA	None	Biosynthesis: phenylalanine, tyrosine
AT-3	70,000[c]	Labile[c]	Labile[c]		GLU ≫ ASP = LEU	PPY > PPA > HPP	Induction[c]	Catabolism: phenylalanine, tyrosine
AT-4	200,000[c]	Stable[c]			GLU = LEU > ASP	PPY = PPA > HPP	Repression[c]	Biosynthesis: phenylalanine, tyrosine
AT-5	200,000	Stable[c]			LEU > GLU	PPY > HPP > PPA	None	Branched chain amino acid metabolism

[a] See Figs. 4 and 5 for detailed studies of aminotransferases AT-1 and AT-2.
[b] Deduced from data given in Table II.
[c] See Ref. 4.

mutagenesis could be separated by recombination in order to assess the subtle effects of individual mutant deficiencies within the complex metabolic arrangement of *P. aeruginosa*.

The Family of Aromatic Aminotransferases in P. aeruginosa—The five species of aminotransferase studied are referred to as "aromatic" aminotransferases only because they are all capable of reaction with prephenate, phenylpyruvate, and 4-hydroxyphenylpyruvate *in vitro*. As indicated in Table IV, we suggest that only enzyme AT-2 and AT-4 are engaged in aromatic biosynthesis in wild type under normal conditions. This is based upon the phenotype of mutant NP-72 in the case of enzyme AT-2 and upon the repression control of enzyme AT-4. Enzyme AT-3 must serve aromatic catabolism ordinarily since it is inducible. Enzyme AT-5 is likely to be a branched chain aminotransferase that may function in a biosynthetic direction, a catabolic direction, or both.

Relative differences in aminotransferase abilities to function with aromatic pathway keto acids are indicated in Table IV. For example, enzyme AT-2 utilizes all three keto acids about equally, in contrast to enzyme AT-1 which utilizes prephenate poorly. Although glutamate was always effective as an amino donor substrate with each aminotransferase enzyme, the relative ability of other amino acids to serve as substrate varied markedly. For example, L-aspartate was very effective with enzyme AT-2 but very unreactive with enzyme AT-3. The reactivity of enzyme AT-4 with L-leucine and its molecular weight may suggest an ancient evolutionary tie between enzyme AT-4 and AT-5.

REFERENCES

1. Jensen, R. A., and Calhoun, D. H. (1981) *Crit. Rev. Microbiol.* **8,** 229–266
2. Weigent, D. A., and Nester, E. W. (1976) *J. Bacteriol.* **128,** 202–211
3. Gelfand, D. H., and Steinberg, R. A. (1977) *J. Bacteriol.* **130,** 429–440
4. Patel, N., Stenmark-Cox, S. L., and Jensen, R. A. (1978) *J. Biol. Chem.* **253,** 2972–2978
5. Patel, N., Pierson, D. L., and Jensen, R. A. (1977) *J. Biol. Chem.* **252,** 5839–5846
6. Fazel, A. M., and Jensen, R. A. (1979) *J. Bacteriol.* **140,** 580–587
7. Holloway, B. W. (1955) *J. Gen. Microbiol.* **13,** 572–581
8. Calhoun, D. H., and Feary, T. W. (1969) *J. Bacteriol.* **97,** 210–216
9. Calhoun, D. H., and Jensen, R. A. (1972) *J. Bacteriol.* **109,** 365–372
10. Metzenberg, R. L., and Mitchell, H. K. (1956) *Arch. Biochem. Biophys.* **64,** 51–56
11. Miller, J. V., and Thompson, E. B. (1972) *Anal. Biochem.* **47,** 487–494
12. Rubin, J. L., and Jensen, R. A. (1979) *Plant Physiol. (Bethesda)* **64,** 727–734
13. Whitaker, R. J., Byng, G. S., Gherna, R. L., and Jensen, R. A. (1981) *J. Bacteriol.* **145,** 752–759
14. Byng, G. S., Whitaker, R. J., Gherna, R. L., and Jensen, R. A. (1980) *J. Bacteriol.* **144,** 247–257
15. Whitaker, R. J., Byng, G. S., Gherna, R. L., and Jensen, R. A. (1981) *J. Bacteriol.* **147,** 526–534
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
17. Gibson, F. (1970) *Methods Enzymol.* **17A,** 362
18. Dayan, J., and Sprinson, D. B. (1970) *Methods Enzymol.* **17A,** 559
19. Zamir, L. O., Jensen, R. A., Arison, B. H., Douglas, A. W., Albers-Schonber, G., and Bowen, J. R. (1980) *J. Am. Chem. Soc.* **102,** 4499–4504
20. Durham, D. R., and Phibbs, P. V. (1982) *J. Bacteriol.* **149,** 534–541
21. Jensen, R. A., Calhoun, D. H., and Stenmark, S. L. (1973) *Biochim. Biophys. Acta* **293,** 256–268
22. Byng, G. S., Kane, J. F., and Jensen, R. A. (1982) *Crit. Rev. Microbiol.* **9,** 227–252
23. Rudman, D., and Meister, A. (1953) *J. Biol. Chem.* **200,** 591–604