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Channel-Shuttle Mechanism for the Regulation of Phenylalanine and Tyrosine Synthesis at a Metabolic Branch Point in *Pseudomonas aeruginosa*

DAVID H. CALHOUN,[1] DUANE L. PIERSON, AND ROY A. JENSEN
Department of Microbiology, Baylor College of Medicine, Houston, Texas 77025

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A bifunctional protein complex was partially purified from *Pseudomonas aeruginosa*. Catalytic activities for chorismate mutase and prephenate dehydratase coeluted from gel filtration and DEAE-cellulose chromatography columns. The protein complex had a molecular weight of approximately 134,000, as determined by gel filtration. In crude extracts or in partially purified preparations about one-half of the chorismate utilized by the complex is converted to phenylpyruvate, and the other half accumulates as prephenate. The chorismate mutase activity is strongly product-inhibited by prephenate, competitively with chorismate. Accordingly, the first reaction of the complex can be sufficiently retarded by prephenate so that all of the reaction product is phenylpyruvate. Chorismate mutase activity is also competitively inhibited by phenylalanine. Although phenylalanine is effective at low concentrations, maximal inhibition is only 50 to 60%. Inhibition of chorismate mutase by phenylalanine was completely lost after gel filtration. The prephenate dehydratase activity of the protein complex is nearly completely inhibited by 0.1 mM phenylalanine in either crude extracts or partially purified preparations. A second species of prephenate dehydratase was separated from the prephenate dehydratase-chorismate mutase aggregate by gel filtration or anion exchange chromatography. The second prephenate dehydratase had an estimated molecular weight of 76,000, a high affinity for prephenate, and was insensitive to feedback inhibition by phenylalanine. The physiological role of the latter enzyme is uncertain. The other regulatory enzymes of tyrosine and phenylalanine biosynthesis, prephenate mutase aggregate by gel filtration or anion exchange chromatography. The other regulatory enzymes of tyrosine and phenylalanine biosynthesis, prephenate dehydrogenase (molecular weight of 120,000) and 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthetase (molecular weight of 52,000), elute from Sephadex G-100 columns as fractions which are distinct from both the chorismate mutase-prephenate dehydratase complex and from the low-molecular-weight species of prephenate dehydratase. A shuttle mechanism governing the metabolic fate of prephenate (to phenylalanine or to tyrosine) is proposed in the context of a model which also accommodates several previously puzzling findings: (i) the dominating role of tyrosine in the control of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthetase and (ii) the lack of feedback control of prephenate dehydrogenase by tyrosine.

In microorganisms an increasing number of examples are being documented of protein complexes carrying two or more related enzyme activities (12). These complexes implicate the importance of spatial and organizational features in the regulation of enzyme activities. When protein complexes mediate reactions at metabolic branch points, they provide a biochemical basis for channeling devices. Channeling refers to the preferential direction of a

[1] Present address: College of Medicine, University of California at Irvine, Irvine, Calif. 92664.

substrate into one of the divergent pathways leading from a metabolic branch point.

The microbial synthesis and regulation of aromatic amino acids have been reviewed recently (11, 19); the pathway for tyrosine and phenylalanine biosynthesis provides examples of many of the multienzyme complexes. In *Bacillus subtilis* three molecular forms of chorismate mutase were fractionated (20); one of these was complexed with 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthetase, the initial enzyme of aromatic biosynthesis. Two distinct forms of chorismate mutase exist in *Salmonella typhimurium*, *Aerobacter aerogenes*, and *Escherichia coli;* one complexed with prephenate dehydratase and the other complexed with prephenate dehydrogenase (6–8, 22). In these cases, the enzyme activities of the chorismate mutase-prephenate dehydratase complex are feedback inhibited by phenylalanine, whereas the activities of the chorismate mutase-prephenate dehydrogenase complex are controlled by tyrosine. Because the pseudomonads are an important microbial group, it is of interest to compare their regulation of aromatic biosynthesis with that of other major microbial groups. *Pseudomonas aeruginosa* was an appropriate choice as a representative species because it constitutes an experimental system in which genetic techniques have been developed (14).

MATERIALS AND METHODS

Growth conditions and extract preparation. *P. aeruginosa* strain 1 was originally obtained from B.W. Holloway (13). Growth conditions and the preparation of extracts were previously described (13). Unless otherwise indicated, crude extracts were dialyzed overnight at 4 C against 1,000 volumes of 50 mM potassium phosphate buffer, *p*H 8.0.

Chorismate mutase assay. Chorismate mutase activity was determined by measuring either the rate of chorismate disappearance (18) or the rate of phenylpyruvate appearance. For the former method the rate of chorismate utilization was monitored continuously in a 1.0-ml cuvette at 274 nm in a Gilford model 240 recording spectrophotometer with the temperature of the reaction chamber thermostatted at 37 C. The rate of disappearance of chorismate was constant for at least 10 min. The molar extinction coefficient for chorismate was taken to be 2,630 (9). The reaction mixture contained 50 mM potassium phosphate buffer, *p*H 8.0, and the indicated amounts of enzyme and substrate. Appropriate controls lacking substrate or enzyme were also included.

When the formation of both reaction products, phenylpyruvate and prephenate, was measured, a reaction mixture volume of 0.25 ml was incubated at 37 C for 10 min. The reaction was terminated by the addition of 0.25 ml of 1.0 N HCl and incubated an additional 10 min at 37 C. The absorbance at 320 nm was determined immediately after the addition of 1.50 ml of 1.0 N NaOH. The activity obtained by measuring the disappearance of chorismate was within 4% of values obtained by measuring the appearance of the resection products (see Results). The acid treatment converts any prephenate present to phenylpyruvate. Accordingly, omission of the acid treatment step resulted in a valid assay for overall conversion of chorismate to phenylpyruvate. By calculation, the difference between the assay results by using the two procedures gives the amount of free prephenate formed. The concentration of phenylpyruvate was estimated by relating the absorbance measured at 320 nm to a molar extinction coefficient of 17,500 (6).

The activity of chorismate mutase in crude extracts is a linear function of protein concentration in the range of about 200 to 800 μg/ml. Most chorismate mutase assays were performed with 250 to 350 μg of extract protein per ml. Chorismate mutase activity is stable for at least several weeks in crude extracts. Partially purified enzyme preparations obtained from ion exchange chromatography lost about 90% of the initial activity within 24 hr. Partially purified preparations obtained from gel filtration separations were stable at 4 C for at least several days; freezing resulted in inactivation.

Prephenate dehydratase assay. The 0.5-ml volume reaction mixture contained the indicated amounts of enzyme and substrate. The reaction was terminated by the addition of 1.5 ml of 1.0 N NaOH. Phenylpyruvate concentrations were estimated by the increment of absorbance at 320 nm. The reaction rates were a linear function of time for at least 20 min. When prephenate dehydratase assays were performed on Sephadex G-100 or diethylaminoethyl (DEAE)-cellulose column eluates, the reaction mixture contained a 0.15-ml column fraction and 0.5 μmole of potassium prephenate in a total volume of 0.2 ml. After 10 min of incubation at 37 C, the reaction was stopped by the addition of 1.8 ml of 1.0 N NaOH, and the phenylpyruvate concentration was determined as described above.

Prephenate dehydratase I, like chorismate mutase, is stable in crude extracts but tends to lose activity after partial purification. In contrast, prephenate dehydratase II is quite stable.

Sephadex G-100 gel filtration. Extracts were prepared from cells harvested from a 1,000-ml culture grown in a 2,800-ml Fernbach flask. The extract protein obtained was concentrated by slowly adding 5 g of solid ammonium sulfate per 10 ml of crude extract, with gentle mixing. The extract was allowed to stand at 4 C for an additional 5 min and then was centrifuged for 20 min at 39,000 × *g*. The precipitate was dissolved in 2 to 3 ml of 50 mM potassium phosphate, *p*H 7.4. Approximately 25 to 30 mg of protein was applied to a Sephadex G-100 column (2.8 by 80 cm) previously equilibrated with the same buffer, and fractions of 2.5 ml were collected at 4 C. Estimates of molecular weight by gel filtration were determined by the method of Andrews (1) by using the following protein standards (Mann Biochemicals)

to calibrate the column: horse heart cytochrome c, rabbit muscle myoglobin, human gamma globulin fraction II, and bovine serum albumin (monomeric and dimeric forms). The void volume was determined with blue dextran.

The activity profile of chorismate mutase from the column was located as a single peak coincident with prephenate dehydratase I. Protein concentration was estimated by determining the optical density at 280 nm of undiluted column fractions. DAHP synthetase was assayed by the procedure of Srinivasan and Sprinson (24) as modified by Jensen and Nester (17). The reaction mixture contained 0.1 ml of column eluate, 0.6 μmole of erythrose 4-phosphate, 0.6 μmole of phosphoenolpyruvate, and 10 μmoles of potassium phosphate buffer, pH 7.4, in a total volume of 0.2 ml. The reaction was continued at 37 C for 10 min. Prephenate dehydrogenase activity was measured by monitoring the increase in fluorescence of nicotinamide adenine dinucleotide, reduced form (NADH), formed in the reaction (4) by use of an Aminco-Bowman recording spectrophotofluorometer. The reaction mixture contained 0.15 ml of column fraction, 0.1 μmole of NAD, 0.5 μmole of potassium prephenate, and 50 μmoles of tris(hydroxymethyl)aminomethane buffer, pH 8.0, in a total volume of 1.0 ml. The reaction was started by adding potassium prephenate to a reaction mixture previously warmed to 37 C. The reaction rate was constant with time for at least 10 min. The fluorescence of NADH was measured at an excitation of 340 nm and an emission of 460 nm (both uncorrected). The velocity is expressed as nmoles of NADH generated per 10 min of incubation at 37 C.

Biochemicals. Chorismate was isolated as the barium salt from culture supernatant fractions of *Aerobacter aerogenes* 62-1 as described by Gibson (10). Barium prephenate was prepared from barium chorismate as described by Gibson (10). Barium chorismate (95% pure) and barium prephenate (91% pure) were converted to the corresponding potassium salts by addition of a twofold molar excess of potassium sulfate. The purity of chorismate preparations was a critical factor in the outcome of certain experiments. For example, a chorismate preparation obtained from Sigma was contaminated with sufficient prephenate to give the apparent result that no free prephenate was formed by chorismate mutase as a reaction product and that all of the reaction product was phenylpyruvate (see Fig. 6 and 7).

Erythrose 4-phosphate, phosphoenolpyruvate, and blue dextran were purchased from Sigma Chemical Co. Amino acids were purchased from Calbiochem. Other chemicals were of the highest purity commercially available and were used without further purification.

RESULTS

Inhibition specificities of chorismate mutase. Chorismate mutase activity was inhibited by both phenylalanine and prephenate in crude extracts. Inhibition by both phenylalanine and prephenate was competitive with chorismate (Fig. 1). In these preparations the

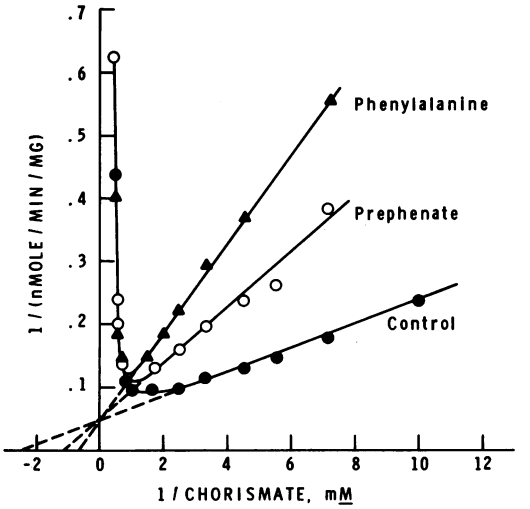

FIG. 1. *Double reciprocal plot showing the competitive inhibitions of chorismate mutase activity by phenylalanine and prephenate. The rate of chorismate disappearance was monitored continuously at 37 C in a cuvette containing 336 μg of protein obtained from a crude extract of strain 1, 50 μmoles of potassium phosphate buffer, pH 8.0, 0.01 μmole of phenylalanine (when present) or 0.25 μmole of potassium prephenate (when present), and the indicated concentration of potassium chorismate in a total volume of 1.0 ml. The apparent substrate inhibition observed at high concentrations of potassium chorismate in this experiment was due to chemical contamination by prephenate, present at a level of approximately 4% of the chorismate (see Materials and Methods).*

apparent Michaelis constant was 0.41 mM, and the apparent maximum velocity was 20 nmoles per minute per milligram of protein. At the final concentrations of prephenate (0.25 mM) and phenylalanine (0.01 mM) used in the experiment shown in Fig. 1 the apparent Michaelis values were increased to 0.83 and 1.43 mM, respectively.

Inhibition of chorismate mutase activity by L-phenylalanine was far from complete, even at high inhibitor concentrations (Fig. 2). Although an inhibition of 50% can easily be demonstrated at phenylalanine concentrations as low as 0.01 mM, inhibition does not exceed 60% in the presence of as much as 1 mM phenylalanine. At saturating concentrations of substrate, maximal inhibition occurs at about 0.1 mM phenylalanine. The inset of Fig. 2 also illustrates inhibition by the phenylalanine analogue, β-2-thienylalanine. The inhibition curve obtained in the presence of thienylalanine was similar to the phenylalanine inhibition curve, except that thienylalanine was less effective on a molar

basis by a factor of 10 to 15. Other aromatic amino acids, or combinations of them, were tested as inhibitors of chorismate mutase. The results given in Table 1 show that at 0.1 mM concentrations, neither tyrosine nor tryptophan was inhibitory. No synergistic effects were noted when various amino acid combinations were used. The presence of tyrosine or tryptophan, or both, in the assay mixture did not affect the inhibition of chorismate mutase by phenylalanine. After partial purification by either gel filtration or anion exchange chromatography, the chorismate mutase activity was insensitive to inhibition by phenylalanine.

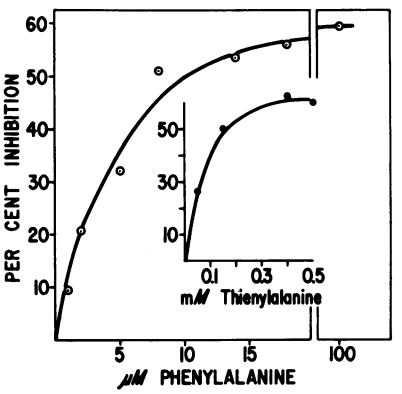

FIG. 2. *Inhibition of chorismate mutase activity by L-phenylalanine and by DL-β-2-thienylalanine in a crude extract prepared from strain 1 grown in minimal salts-glucose medium. The rate of chorismate disappearance was measured continuously at 37 C in a cuvette containing 0.5 μmole of potassium chorismate, 185 μg of protein, 50 μmoles of potassium phosphate buffer, pH 8.0, and the indicated concentration of phenylalanine or thienylalanine (inset) in a total volume of 1.0 ml. The values on the ordinate scales of both curves are expressed as per cent inhibition. The values were calculated by relating reaction velocities measured in the presence of inhibitor to control reaction mixtures lacking inhibitor. The control reaction mixture exhibited a specific activity of 17 nmoles per minute per milligram of extract protein.*

TABLE 1. *Effect of aromatic amino acids on chorismate mutase activity*[a]

Amino acid	Specific activity	% Inhibition
None	12.4	
Phenylalanine	6.1	51
Tyrosine	13.4	0
Tryptophan	13.4	0
Phenylalanine + tyrosine	6.5	48
Phenylalanine + tryptophan	6.5	48
Tyrosine + tryptophan	13.3	0
Phenylalanine + tyrosine + tryptophan	5.9	52

[a] The reaction mixture contained 354 μg of protein from a crude extract prepared from strain 1 grown in minimal salts-glucose medium at 37 C, 0.42 μmole of chorismate, and 50 μmoles of potassium phosphate buffer, pH 8.0, in a reaction volume of 1.0 ml. Amino acids were present at 0.1 mM. Specific activity is expressed as nanomoles of chorismate utilized per minute per milligram of protein.

Lack of repression control. Cultures were grown in the presence of aromatic amino acids to determine whether the activity present in cells grown in minimal salts-glucose medium was repressed by end products (Table 2). The specific activity of chorismate mutase was not affected by growth with aromatic amino acids. This result, however, does not exclude the possibility that the activity might derepress after starvation for aromatic end products in certain aromatic auxotrophs.

The possibility was considered that isoenzymes with differing sensitivities to enzyme inhibition may be synthesized under different growth conditions and that total specific activity measured under different growth conditions might be fortuitiously similar. Accordingly, the extracts prepared from cells grown under potential conditions of repression were also tested for inhibition by phenylalanine (Table 2). No differences in the degree of inhibition were apparent, suggesting the absence of isoenzymes of chorismate mutase. In addition, chorismate mutase activity eluted from Sephadex G-100 and DEAE-cellulose columns as a single component of the protein eluate (see below).

Cofractionation of chorismate mutase and prephenate dehydratase I. The activity of chorismate mutase eluted from a Sephadex G-100 column as a single component (Fig. 3). However, this activity was coincident with one of two molecular weight species of prephenate

TABLE 2. *Chorismate mutase activity in cells grown with aromatic amino acids*[a]

Growth supplement (3 mM)	Specific activity	% Inhibition by 0.1 mM phenylalanine
None	15.0	42
Tyrosine	17.2	42
Phenylalanine	15.7	37
Phenylalanine + tyrosine + tryptophan	12.9	42

[a] The reaction mixture contained 250 to 300 μg of crude extract protein prepared from strain 1, 0.8 μmole of potassium chorismate, and 50 μmoles of potassium phosphate buffer, pH 8.0, in a reaction volume of 1.0 ml. Specific activity is expressed as nanomoles of chorismate utilized per minute per milligram of protein.

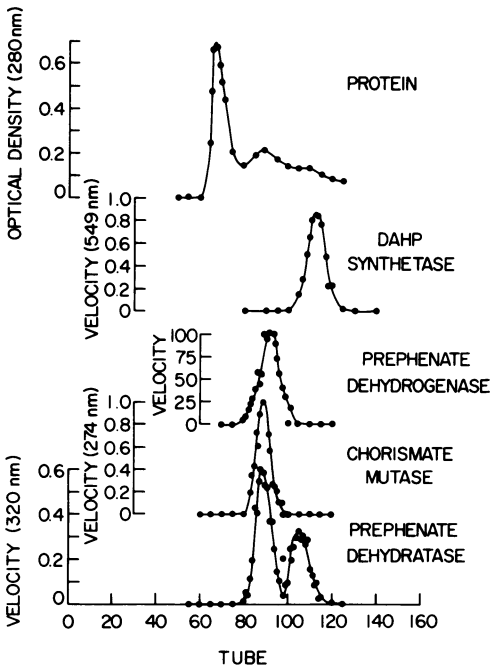

FIG. 3. *Fractionation of aromatic amino acid pathway enzymes by Sephadex G-100 gel filtration. A 2-ml amount of an ammonium sulfate precipitate from a crude extract prepared from strain 1 (ca. 25 mg of protein) was applied to a column previously equilibrated with 50 mM potassium phosphate buffer, pH 7.4. Fractions of 2.5 ml were collected at a flow rate of 20 ml per hour and assayed for the indicated activities as described. Estimates of molecular weights were determined from a standard curve based upon the elution of horse heart cytochrome c, rabbit muscle myoglobin, human gamma globulin fraction II, and bovine serum albumin (monomeric and dimeric states). The larger molecular weight species of prephenate dehydratase (prephenate dehydratase I) has a peak elution position at tube 88, whereas prephenate dehydratase II has a peak elution position at tube 105. All enzyme activities are given on the ordinate scales as velocity (expressed as activity per 10 min; see Materials and Methods).*

dehydratase. The chorismate mutase-prephenate dehydratase aggregate elutes at a position corresponding to a molecular weight of 134,000. The second prephenate dehydratase species (denoted prephenate dehydratase II) has an estimated molecular weight of 76,000. The eluate fractions corresponding to each of the two molecular weight species of prephenate dehydratase activity were pooled, concentrated by ammonium sulfate precipitation, resuspension in buffer, and passed through the Sephadex G-100 column a second time. Each molecular weight species eluted the second time as a single, sharp peak corresponding exactly to the position of the original eluate profile. Hence, there is no evidence for an association-dissociation relationship of prephenate dehydratase I and prephenate dehydratase II proteins which could be in equilibrium with one another, as occurs with the different species of chorismate mutase proteins in *B. subtilis* (20). However, the demonstration of such a relationship might require the mediation of small molecules such as phenylalanine, chorismate, or prephenate (23).

As shown in Fig. 3, both chorismate mutase-prephenate dehydratase I (molecular weight 134,000) and prephenate dehydratase II (molecular weight 76,000) are separable from DAHP synthetase (approximate molecular weight 52,000), the first enzyme in the aromatic amino acid pathway, and from prephenate dehydrogenase (approximate molecular weight 120,000), the first enzyme in the pathway leading to tyrosine.

The cofractionation of chorismate mutase and prephenate dehydratase I activities as well as the separate recovery of prephenate dehydratase II was also observed after elution from a column packed with DEAE-cellulose. Conceding the difficulty of accurately relating the enzyme activities found in crude extracts with those recovered after partial purification, chorismate mutase-prephenate dehydratase I was recovered at >90% from gel filtration columns and at about a 75% yield after DEAE chromatography. Prephenate dehydratase II recovery was estimated to be 100%. In both of the fractionations shown in Fig. 3 and 4, prephenate dehydratase I was quite sensitive to inhibition by phenylalanine whereas prephenate dehydratase II activity was not appreciably affected by phenylalanine. (The relatively large proportion of prephenate dehydratase II seen in Fig. 4 is due to the instability of prephenate dehyratase I under these conditions.) The data in Fig. 5 show that prephenate dehydratase II activity was inhibited no more than 15 to 20% at 0.2 mM concentrations (or greater) of phenylalanine, whereas prephenate dehydratase I activity (inset) was totally abolished in the presence of 0.2 mM phenylalanine. The same relative sensitivities to phenylalanine inhibition were found with DEAE-cellulose fractions as with Sephadex G-100 column fractions. The two prephenate dehydratases are qualitatively different in affinity for prephenate; prephenate dehydratase II approaches saturation at 0.5 mM prephenate, whereas prephenate dehydratase I maintains first order reactivity at substrate concentrations as high as 2.5 mM prephenate (Fig. 5). Because phenylalanine

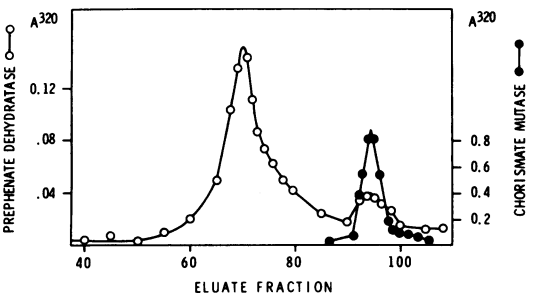

FIG. 4. *DEAE-cellulose chromatography of a crude extract from strain 1. A 2-ml amount of an extract concentrated by ammonium sulfate precipitation and containing 40 mg of protein was dialyzed against 10 mM potassium phosphate buffer, pH 7.0, and applied to a 1.5 by 15 cm column of DE52 resin (Whatman) equilibrated with 10 mM potassium phosphate buffer, ph 7.0. Fractions of 2.5 ml were eluted with a linear gradient of NaCl (0 to 0.5 M) at a flow rate of 0.5 ml per min. Each reservoir contained a 250 ml volume. Eluates were assayed as described in Materials and Methods. Velocities for chorismate mutase and prephenate dehydratase activities are expressed as Δ absorbancy at 320 nm per 10 min at 37 C. The phenylalanine-insensitive prephenate dehydratase II elutes at fraction 70, whereas the phenylalanine-inhibitable prephenate dehydratase I elutes with chorismate mutase at fraction 95.*

selectively inhibits the activity of prephenate dehydratase I, the relative proportions of the two activities in crude extracts can be estimated fairly accurately by assay in the presence and in the absence of phenylalanine. In crude extracts 68% of the total prephenate dehydratase activity is inhibited by 0.1 mM phenylalanine at a substrate concentration of 1.0 mM prephenate (Table 3). The activity in crude extracts represents the sum of the activities of prephenate dehydratase I and the smaller molecular weight prephenate dehydratase II. The 32% of the total activity not inhibitable by phenylalanine represents the activity of the phenylalanine-insensitive prephenate dehydratase II present in crude extracts at this substrate concentration. The other aromatic amino acids, tyrosine and tryptophan, do not inhibit prephenate dehydratase activity in crude extracts (Table 3) or in partially purified preparations. The total level of prephenate dehydratase activity found in crude extracts is not influenced (Table 4) by growth of the cultures in the presence of aromatic amino acids. Hence, enzyme levels either are not regulated by repression control or repression is maximal under conditions of growth in minimal medium.

Overall reaction catalyzed by chorismate mutase-prephenate dehydratase I. When chorismate is utilized by chorismate mutase, a mixture of two reaction products is formed. Prephenate and phenylpyruvate are formed in a ratio of about 45:55 in reaction mixtures containing crude extract under the conditions of assay used.

The reaction mixture contained 352 μg of crude extract (prepared from strain 1 grown at 37 C on minimal salts-glucose medium), 1.0

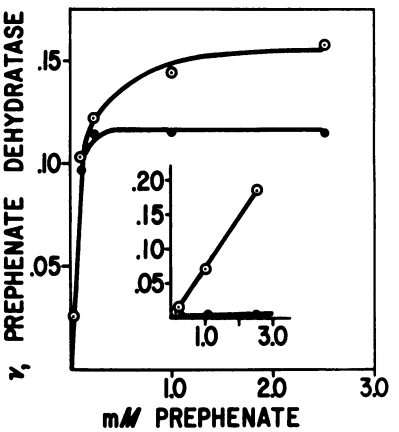

FIG. 5. *Effect of phenylalanine on the activities of the two partially purified species of prephenate dehydratase. The Sephadex G-100 fractions containing the peak activities for prephenate dehydratases I and II (Fig. 4) were pooled (ca. 20 ml for each activity), and the protein was precipitated with ammonium sulfate (70% saturation) and resuspended in 2.0 to 3.0 ml of 100 mM potassium phosphate buffer, pH 7.0. Reaction mixtures contained 0.15 ml of a partially purified prephenate dehydratase I or II eluate fraction, the indicated concentrations of potassium prephenate, 0.05 μmole of phenylalanine (when present), and 15 μmoles of potassium phosphate buffer, pH 7.0, in a final volume of 0.25 ml. The reaction was terminated after 10 min at 37 C. Curves obtained with prephenate dehydratases II and I are represented on the left and in the inset on the right, respectively. Symbols: no phenylalanine present, ⊙; phenylalanine present, ●.*

TABLE 3. *Effect of aromatic amino acids on the activity of prephenate dehydratase in crude extracts*[a]

Inhibitor	Specific activity	% Inhibition
None	19.7	
Phenylalanine	6.3	68
Tyrosine	19.7	0
Tryptophan	21.0	0

[a] The reaction mixture contained 165 μg of protein, 25 μmoles of Tris-hydrochloride buffer, pH 8.0, and 0.5 μmole of potassium prephenate in a total volume of 0.5 ml. Specific activity is expressed as nanomoles of phenylpyruvate formed per minute per milligram. The reaction was terminated after 20 min of incubation at 37 C. Inhibitors were present at a final concentration of 0.1 mM.

TABLE 4. *Effect of aromatic amino acids on the synthesis of prephenate dehydratase(s)*[a]

Growth supplement (3 mM)	Specific activity
None	19.5
Phenylalanine	20.4
Tyrosine	24.4
Phenylalanine + tyrosine + tryptophan	22.4

[a] The reaction mixture contained 0.5 μmole of potassium prephenate, 185 to 240 μg of protein, and 25 μmoles of tris(hydroxymethyl)aminomethane-hydrochloride buffer, *p*H 8.0, in a total volume of 0.5 ml. Specific activity is expressed as nmoles of phenylpyruvate formed per minute per milligram of protein. The reaction was terminated after 20 min of incubation at 37C.

μmole of chorismate, and 50 μmoles of potassium phosphate buffer, *p*H 8.0, in a total volume of 1.0 ml. Chorismate utilization was measured as rate of decrease in absorbance at 274 nm. The overall conversion of chorismate to phenylpyruvate was measured directly by reading phenylpyruvate absorbance at 320 nm in 0.75 N NaOH. Total product formation was measured as phenylpyruvate in 0.75 N NaOH at 320 nm after acid conversion of any prephenate to phenylpyruvate by incubation for 10 min at 37 C in 0.5 N HCl. Prephenate concentration was calculated as the difference between phenylpyruvate and total product (i.e., prephenate plus phenylpyruvate). The substrate-product relationships were as follows: chorismate utilized, 37.4 nmoles/10 min; prephenate formed, 16.1 nmoles/10 min; and phenylpyruvate, 19.8 nmoles/10 min.

Approximately equal levels of prephenate and phenylpyruvate formation by the chorismate mutase-prephenate dehydratase I aggregate that were found in crude extracts also characterize the partially purified chorismate mutase-prephenate dehydratase I (compare ordinate values of the two curves shown in Fig. 6). As previously shown (Fig. 1) the chorismate mutase reaction is product-inhibited by prephenate. As increasing concentrations of prephenate are added to the reaction mixture (Fig. 6), the amount of prephenate measured as reaction product diminishes and is nil at an added prephenate concentration of about 0.4 mM. Over this concentration range of prephenate, phenylpyruvate formation almost doubles. The increased phenylpyruvate formation is accounted for most easily by the additional substrate (prephenate) available for the second reaction of the complex.

The second reaction (i.e., prephenate dehydratase I) is strongly inhibited by phenylalanine. The first reaction (i.e., chorismate mutase) is more weakly inhibited in crude extracts. One would expect that prephenate would accumulate as the sole reaction product of chorismate mutase-prephenate dehydratase I in the presence of phenylalanine. This expectation is fulfilled in the data shown in Fig. 7. In the absence of phenylalanine 48% of the reaction product was prephenate and 52% was phenylpyruvate. In the presence of 0.33 mM phenylalanine 98% of the reaction product was prephenate and only 2% was phenylpyruvate.

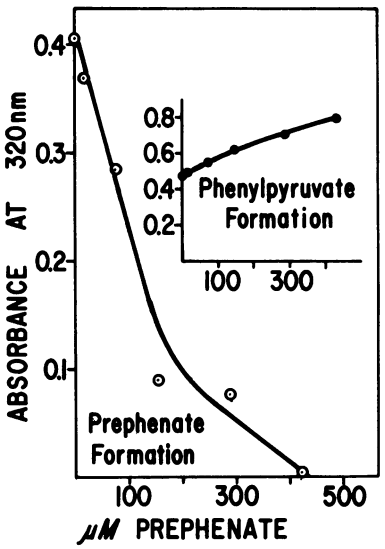

FIG. 6. *Effect of added prephenate during chorismate mutase catalysis. Variable concentrations of potassium prephenate were added to the reaction mixture as indicated along the abscissa scale. Under identical reaction conditions phenylpyruvate formation was also followed (shown in the inset). The major abscissa and ordinate labels also apply to the inset. Prephenate and phenylpyruvate concentrations were estimated according to the methods given in Materials and Methods. An absorbance value of 0.1 at 320 nm corresponds to 5.7 nmoles of prephenate or phenylpyruvate present per ml of base-treated reaction mixture after a 10-min reaction at 37 C; this corresponds to a prephenate concentration of 49.2 μM in the original reaction volume at the termination of the reaction. The reaction mixtures each contained a 2.2 mM concentration of potassium chorismate and 20 μg of protein from the peak fraction of chorismate mutase-prephenate dehydratase I eluted from Sephadex G-100 as described in Fig. 3.*

DISCUSSION

Prephenate dehydratase II. Prephenate dehydratase II, one of two species of prephenate dehydratase separated by gel filtration and DEAE-cellulose chromatography in *P. aeruginosa*, has a molecular weight of approxi-

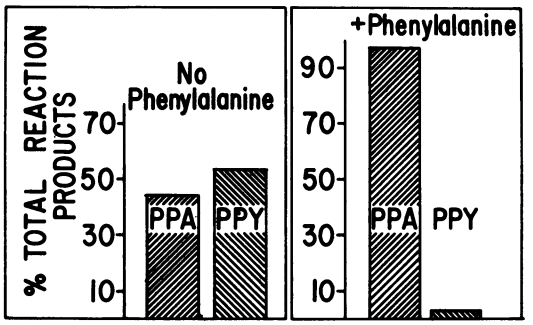

FIG. 7. *Accumulation of prephenate as the sole reaction product of the chorismate mutase-prephenate dehydratase I protein in the presence of phenylalanine. The ordinate scales indicate the fraction of prephenate (PPA) or phenylpyruvate (PPY) formed as the per cent (on a molar basis) of the sum of prephenate and phenylpyruvate. Prephenate and phenylpyruvate concentrations were measured as described in Materials and Methods. The levels of prephenate and phenylpyruvate are shown in histogram form and represent an average of several experiments in which the concentration of chorismate was varied from 1.1 to 4.5 mM. No appreciable differences in results were noted within this substrate range. Phenylalanine was present (right graph) at a final concentration of 0.33 mM. The actual amounts of prephenate and phenylpyruvate formed in a representative experiment done at 3.4 mM potassium chorismate were 6.64 and 7.50 nmoles per 0.4 ml of reaction mixture per minute at 37 C (left), respectively, and 14.12 and 0.24 nmoles per 0.4 ml of reaction mixture per minute at 37 C (right), respectively. The reaction mixtures contained 20 μg of protein from the peak tube of a Sephadex G-100 eluate obtained as described in Fig. 3.*

mately 76,000 and shows a relatively high affinity for prephenate. It could represent a dissociation or interconversion product of prephenate dehydratase I. Because it is not subject to feedback inhibition by phenylalanine, it is tentatively assumed that prephenate dehydratase II lacks a physiological role in aromatic biosynthesis. Prephenate dehydratase II could be an in vitro fragment possessing no meaningful physiological counterpart.

The multiplicity of prephenate dehydratase in *P. aeruginosa* is reminiscent of some characteristics of prephenate dehydratase in other microorganisms. In *A. aerogenes*, a second minor prephenate dehydratase, distinct from the prephenate dehydratase associated with an isoenzymic chorismate mutase, was eluted from DEAE-cellulose (6). This second prephenate dehydratase activity was not inhibited by phenylalanine and was present in phenylalanine auxotrophs lacking the chorismate mutase (P)-prephenate dehydratase complex. In *B. subtilis*, Coats and Nester (5) described two molecular forms of prephenate dehydratase with molecular weights of approximately 65,000 and 190,000. In *S. typhimurium*, prephenate dehydratase apparently exists as an equilibrium mixture of monomer and dimer; phenylalanine appears to "shift" the equilibrium in favor of the dimer (21, 23). (In *P. aeruginosa* the molecular weight data are consistent with the possibility that chorismate mutase-prephenate dehydratase I could be a dimer of prephenate dehydratase II.) Additional biochemical and genetic experiments will be required to determine the relationship of prephenate dehydratase II to aromatic amino acid synthesis.

Possible role of phenylalanine hydroxylase. Pseudomonads are unique among microorganisms in their mammalian-like ability to convert phenylalanine to tyrosine via phenylalanine hydroxylase. The existence of active phenylalanine hydroxylase in strain 1 of *P. aeruginosa* would be relevant to the interpretation of much of our data. For example, a single DAHP synthetase inhibitable by tyrosine in *P. aeruginosa* would in fact be appropriate to a pathway in which tyrosine is an end product and phenylalanine is a precursor of tyrosine. Phenylalanine hydroxylase is an inducible, catabolic enzyme which degrades phenylalanine through tyrosine prior to cleavage of the aromatic ring. Stanier et al. (25) surveyed the prevalence of phenylalanine hydroxylase in *P. aeruginosa*. It was found that 28 of 29 strains grew on tyrosine as a sole source of carbon, whereas only seven grew on phenylalanine as a sole source of carbon.

Indeed, it was shown that strain 1 is representative of the major type described by Stanier et al. (25), i.e., it utilizes tyrosine but not phenylalanine as a sole source of carbon (2). Furthermore, auxotrophic mutants blocked in the common portion of the aromatic acid pathway require tyrosine in addition to phenylalanine and tryptophan for growth (2). Based on nutritional data, phenylalanine hydroxylase in strain 1 seems to be slowly inducible over long periods of time in the presence of phenylalanine. Mutant derivatives of wild type can be isolated which can grow on a phenylalanine carbon source. Likewise, spontaneous secondary mutants can be selected from aromatic acid-requiring auxotrophs which can grow without tyrosine but which retain a requirement for phenylalanine and tryptophan. All available evidence suggests that the wild-type organism does not possess significant ability to convert phenylalanine directly to tyrosine under ordinary laboratory conditions of growth. The existence of the biosynthetic prephenate dehydrogenase also argues against the biosynthetic role

of phenylalanine hydroxylase.

Control of aromatic amino acid synthesis by repression. As appears to be common with biosynthetic enzymes in pseudomonads (14), variation of enzyme levels by repression does not appear to constitute an important regulatory device. Thus, DAHP synthetase (15), chorismate mutase-prephenate dehydratase I, prephenate dehydratase II, and prephenate dehydrogenase (D. L. Pierson, *unpublished data*) all exhibit invariant levels of enzyme under various regimens of nutrition. It is interesting that the levels of the member enzymes of the tryptophan pathway are subject to repression in a range which varies by at least a factor of 50 (D. H. Calhoun and D. L. Pierson, *unpublished data*).

The multi-functional protein, a channel to phenylalanine. Prephenate dehydratase I appears to be a bifunctional protein which also catalyzes the chorismate mutase reaction. The protein has a molecular weight of about 134,000. The prephenate dehydratase I activity is very sensitive to feedback inhibition by phenylalanine. Although the associated chorismate mutase activity of this protein is also inhibited by phenylalanine in crude extracts, inhibition does not exceed 50%. The relatively poor affinity of prephenate dehydratase I for prephenate probably reflects an in vivo channeling mechanism in which prephenate is not ordinarily utilized by prephenate dehydratase I as a free intermediate. Seemingly, a single chorismate mutase, which channels prephenate toward phenylpyruvate by virtue of its association with prephenate dehydratase I, would cause starvation for tyrosine precursor molecules. Another undetected chorismate mutase could exist which is labile. Alternatively, phenylalanine inhibition of the prephenate dehydratase I reaction might make free prephenate available as substrate for prephenate dehydrogenase, a possibility which is consistent with our data.

Regulation by a channel-shuttle mechanism. The apparent existence of a single chorismate mutase that is associated with an enzyme converting its product (prephenate) to to a phenylalanine precursor (phenylpyruvate) raises a question about the origin of prephenate for tyrosine synthesis. The scheme shown in Fig. 8 presents a postulated shuttle mechanism which allows prephenate to be diverted toward tyrosine synthesis. The differential sensitivities of prephenate dehydratase I and chorismate mutase to inhibition by phenylalanine is a critical element of the shuttle mechanism. Shuttle mechanisms frequently employ a shuttle which moves back and forth alternately blocking and freeing one or more openings causing some entity to be routed first one way and then another. By analogy the entity is prephenate and the shuttle molecule is phenylalanine, capable of blocking prephenate dehydratase activity when present.

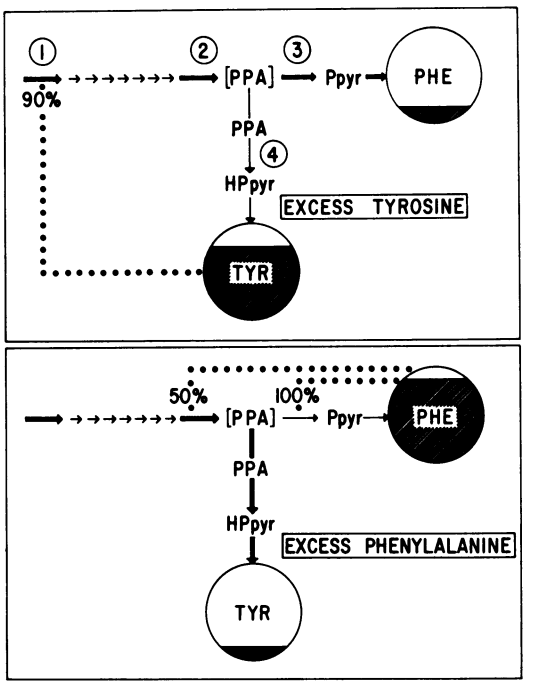

FIG. 8. *Channel-shuttle model for allosteric control of phenylalanine and tyrosine synthesis. Heavy arrows denote main flow route of intermediary metabolites. Dotted lines show feedback inhibition with maximal levels of inhibition indicated in per cent. (Top) Under unbalanced conditions of excess tyrosine (TYR) and limiting phenylalanine (PHE), intermediates are channeled toward phenylalanine synthesis. Tyrosine feedback inhibits (but does not repress) 3 - deoxy - D - arabino - heptulosonate - 7 - phosphate (DAHP) synthetase (denoted 1). Prephenate, in the absence of phenylalanine, tends to be channeled preferentially toward phenylalanine by virtue of the association of chorismate mutase and prephenate dehydratase I (denoted as 2 and 3, respectively). Although prephenate dehydrogenase (denoted 4) is not feedback inhibited by tyrosine, little or no free prephenate (PPA) would be available to prephenate dehydrogenase as substrate. (Bottom) Under unbalanced conditions of excess phenylalanine and limiting tyrosine, prephenate dehydratase I is completely inhibited, and DAHP synthetase is not inhibited. Continued activity of the first reaction, i.e., chorismate mutase, results in the release of free prephenate which then is available as substrate for prephenate dehydrogenase. Abbreviations: Ppyr, phenylpyruvate; HPpyr, p-hydroxyphenylpyruvate.*

It should be noted that the amount of free prephenate formed in the absence of phenylalanine in experiments such as that of Fig. 7 is

about the in vitro maximum, being obtained at saturating concentrations of chorismate. Because substrate concentrations of enzymes in vivo presumably are far below saturation, it seems probable that at least a partial closure of the channel by endogenous phenylalanine is obligatory for the release of prephenate for use as a tyrosine precursor.

When phenylalanine is relatively limiting, the channel route would dominate, and chorismate would be preferentially routed toward phenylalanine synthesis. If tyrosine levels are also relatively limiting, precursor levels would be at a maximum, because the activity of DAHP synthetase is strongly inhibited by tyrosine (15). When intracellular phenylalanine levels are high and tyrosine levels are limiting, the shuttle mechanism operates. Phenylalanine negates the second reaction of chorismate mutase-prephenate dehydratase I, and free prephenate accumulates, serving as substrate for prephenate dehydrogenase and therefore as precursor for tyrosine. Under conditions of excess tyrosine and phenylalanine, tyrosine would decrease precursor formation at the level of DAHP synthetase, whereas phenylalanine would regulate at the level of another branch point, chorismate mutase-prephenate dehydratase. Under balanced conditions of phenylalanine and tyrosine supply, each metabolic branch point would be expected to be under partial control by its cognate effector. The inhibition of chorismate mutase by prephenate may also be of regulatory significance.

Such a control mechanism could account for apparent lack of allosteric control of prephenate dehydrogenase by tyrosine. Excess tyrosine could control tyrosine formation by limiting precursor formation due to allosteric inhibition of DAHP synthetase, especially because precursor limitation would tend to favor the channel, preferentially converting chorismate to phenylpyruvate. A related microorganism, *Comamonas* sp. ATCC 11299a (3), also possesses a prephenate dehydrogenase which is insensitive to feedback inhibition. Such a mechanism seems to provide a reasonable physiological explanation for the dominating role of tyrosine in the control of DAHP synthetase in various pseudomonads (15, 16). In effect, the regulatory signal represented by excess phenylalanine is translated indirectly to another regulatory signal (excess tyrosine) through the shuttle mechanism. Hence, the mechanism would accommodate the partial regulation of DAHP synthetase in the presence of either excess phenylalanine or tyrosine.

Because tyrosine is a very effective inhibitor of DAHP synthetase (15), we assume that the activity of DAHP synthetase is "set" at a high level which ensures adequate precursor formation for other aromatic end products, even in the presence of tyrosine. This is consistent with the facts that tyrosine (to which the cells are permeable) does not act as an inhibitor of growth in *P. aeruginosa* and does not result in the derepression of one or more isoenzymes of DAHP synthetase (as occurs in many microorganisms, for example) (16).

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LITERATURE CITED

1. Andrews, P. 1964. Estimation of the molecular weight of proteins by Sephadex gel-filtration. Biochem. J. **91**:222-233.
2. Calhoun, D. H., and R. A. Jensen. 1972. Significance of altered carbon flow in aromatic amino acid synthesis: an approach to the isolation of regulatory mutants in *Pseudomonas aeruginosa*. J. Bacteriol. **109**:365-372.
3. Cerutti, P., and G. Guroff. 1965. Enzymatic formation of phenylpyruvic acid in *Pseudomonas* sp. (ATCC 11299a) and its regulation. J. Biol. Chem. **240**:3034-3038.
4. Champney, W. S., and R. A. Jensen. 1970. The enzymology of prephenate dehydrogenase in *Bacillus subtilis*. J. Biol. Chem. **245**:3763-3770.
5. Coats, J. H., and E. W. Nester. 1967. Regulation reversal mutation: characterization of end product-activated mutants of *Bacillus subtilis*. J. Biol. Chem. **242**:4948-4955.
6. Cotton, R. G. H., and F. Gibson. 1965. The biosynthesis of phenylalanine and tyrosine; enzymes converting chorismic acid into prephenic acid and their relationships to prephenate dehydrogenase. Biochim. Biophys. Acta **100**:76-88.
7. Cotton, R. G. H., and F. Gibson. 1967. The biosynthesis of tyrosine in *Aerobacter aerogenes:* partial purification of the T protein. Biochim. Biophys. Acta **147**:222-237.
8. Cotton, R. G. H., and F. Gibson. 1968. The biosynthesis of tyrosine in *Aerobacter aerogenes:* evidence for a subunit structure of the protein converting chorismate into 4-hydroxyphenylpyruvate. Biochim. Biophys. Acta **160**:188-195.
9. Edwards, J. M., and L. M. Jackman. 1965. Chorismic acid. A branch point intermediate in aromatic biosynthesis. Aust. J. Chem. **18**:1227.
10. Gibson, F. 1964. Chorismic acid: purification and some chemical and physical studies. Biochem. J. **90**:256-261.
11. Gibson, F., and J. Pittard. 1968. Pathways of biosynthesis of aromatic amino acids and vitamins and their control in microorganisms. Bacteriol. Rev. **32**:465-492.
12. Ginsburg, A., and E. R. Stadtman. 1970. Multienzyme systems. Annu. Rev. Biochem. **39**:429-472.
13. Holloway, B. W. 1955. Genetic recombination in *Pseudomonas aeruginosa*. J. Gen. Microbiol. **13**:572-581.
14. Holloway, B. W. 1969. Genetics of *Pseudomonas*. Bacter-

iol. Rev. **33**:419–443.
15. Jensen, R. A., D. H. Calhoun, and S. L. Stenmark. 1972. Allosteric inhibition of 3-deoxy-*D*-arabino-heptulosonate 7-phosphate synthetase by tyrosine in *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **278**, *in press*.
16. Jensen, R. A., D. S. Nasser, and E. W. Nester. 1967. Comparative control of a branch-point enzyme in microorganisms. J. Bacteriol. **94**:1582–1593.
17. Jensen, R. A., and E. W. Nester. 1966. Regulatory enzymes of aromatic amino acid biosynthesis in *Bacillus subtilis*. I. Purification and properties of DAHP synthetase. J. Biol. Chem. **241**:3365–3372.
18. Kane, J. F., S. L. Stenmark, D. H. Calhoun, and R. A. Jensen. 1971. Metabolic interlock: the role of the subordinate type of enzyme in the regulation of a complex pathway. J. Biol. Chem. **246**:4308–4316.
19. Lingens, F. 1968. The biosynthesis of aromatic amino acids and its regulation. Angew. Chem. Int. Ed. Engl. **7**:350–360.
20. Lorence, J., and E. W. Nester. 1967. Multiple molecular forms of chorismate mutase in *Bacillus subtilis*. Biochemistry **6**:1541–1553.
21. Schmit, J. C., S. W. Artz, and H. Zalkin. 1970. Chorismate mutase-prephenate dehydratase. Evidence for distinct catalytic and regulatory sites. J. Biol. Chem. **245**:4019–4027.
22. Schmit, J. C., and H. Zalkin. 1969. Chorismate mutase-prephenate dehydratase. Partial purification and properties of the enzyme from *Salmonella typhimurium*. Biochemistry **8**:174–181.
23. Schmit, J. C., and H. Zalkin. 1971. Chorismate mutase-prephenate dehydratase. Phenylalanine-induced dimerization and its relationship to feedback inhibition. J. Biol. Chem. **246**:6002–6010.
24. Srinivasan, P. R., and D. B. Sprinson. 1959. 2-Keto-3-deoxy-D-arabo-heptonic acid 7-phosphate synthetase. J. Biol. Chem. **234**:716–722.
25. Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43**:159–271.