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L-Arogenate Is a Chemoattractant Which Can Be Utilized as the Sole Source of Carbon and Nitrogen by *Pseudomonas aeruginosa*†

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L-Arogenate is a commonplace amino acid in nature in consideration of its role as a ubiquitous precursor of L-phenylalanine and/or L-tyrosine. However, the questions of whether it serves as a chemoattractant molecule and whether it can serve as a substrate for catabolism have never been studied. We found that *Pseudomonas aeruginosa* recognizes L-arogenate as a chemoattractant molecule which can be utilized as a source of both carbon and nitrogen. Mutants lacking expression of either cyclohexadienyl dehydratase or phenylalanine hydroxylase exhibited highly reduced growth rates when utilizing L-arogenate as a nitrogen source. Utilization of L-arogenate as a source of either carbon or nitrogen was dependent upon σ^{54}, as revealed by the use of an *rpoN* null mutant. The evidence suggests that catabolism of L-arogenate proceeds via alternative pathways which converge at 4-hydroxyphenylpyruvate. In one pathway, prephenate formed in the periplasm by deamination of L-arogenate is converted to 4-hydroxyphenylpyruvate by cyclohexadienyl dehydrogenase. The second route depends upon the sequential action of periplasmic cyclohexadienyl dehydratase, phenylalanine hydroxylase, and aromatic aminotransferase.

L-Arogenate (α-amino-1-carboxy-4-hydroxy-2,5-cyclohexadiene-1-propanoic acid) (AGN) is a widely used precursor of L-phenylalanine and/or L-tyrosine in nature (15 and references therein). In higher plants, both L-phenylalanine and L-tyrosine are directly derived from AGN in a single step. A diversity of biochemical routings to L-phenylalanine and L-tyrosine, via a variety of AGN–phenylpyruvate–4-hydroxyphenylpyruvate combinations, has been delineated in bacteria.

Pseudomonas aeruginosa belongs to a large group of gram-negative prokaryotes that maintain coexisting pathways whereby AGN and phenylpyruvate or AGN and 4-hydroxyphenylpyruvate can be utilized as alternative precursors of L-phenylalanine and L-tyrosine, respectively (22). These biochemical options are illustrated in Fig. 1. Two separate pathways exist for L-phenylalanine biosynthesis. The primary pathway to L-phenylalanine utilizes a bifunctional protein (P protein) having catalytic domains for chorismate mutase and prephenate dehydratase. The P protein is of ancient origin and is distributed throughout two of the three major superfamilies of gram-negative bacteria (2, 3, 14). A second pathway occupies the periplasmic compartment (10, 30, 31) and employs a monofunctional species of chorismate mutase (AroQ, previously designated CM-F), a periplasmic species of aromatic aminotransferase (AT_p), and cyclohexadienyl dehydratase (PheC). It has been termed the overflow pathway (9) on the basis of the absence of allosteric control by end products and the apparent dependence of L-phenylalanine output upon substrate (chorismate) availability. The broad specificity of PheC allows prephenate conversion to L-phenylalanine via either phenylpyruvate or AGN (Fig. 1).

Cyclohexadienyl dehydrogenase (TyrC), a broad-specificity enzyme, had previously been shown to be the key catalyst responsible for the alternative AGN and 4-hydroxyphenylpyruvate routings (27) to L-tyrosine. The recent discovery (23, 32) that *P. aeruginosa* possesses an inducible multigene operon that includes phenylalanine hydroxylase (PhhA) revealed a third biochemical pathway that can lead to L-tyrosine.

Pseudomonads inhabit a wide array of environmental niches, ranging from soil and water to human hosts, and are able to metabolize a broad range of compounds. *P. aeruginosa* has been shown to utilize most of the commonly occurring amino acids for growth as either sole carbon or nitrogen sources (17). Amino acids have also proven to be particularly effective chemoattractants, and *P. aeruginosa* is attracted to most of the 20 common amino acids (6). Efficient utilization for growth occurs even at low amino acid concentrations. Chemotaxis and motility are widespread and highly conserved capabilities in the eubacteria, indicating their functional importance. Consequently, chemotaxis is believed to be advantageous for bacteria in environments where nutrients are scarce. An organism that can direct its movement toward a desirable nutrient would have an increased chance for survival. Despite the ubiquitous presence of AGN in nature and the consequent likelihood that this amino acid would be a valuable molecule to salvage from the exogenous environment, no studies of chemotaxis or metabolic utilization of AGN have been reported.

In this report, we describe the abilities of *P. aeruginosa* to recognize AGN as an elicitor of chemotaxis and to utilize AGN as a sole source of both carbon and nitrogen. We suggest a primary pathway and a minor pathway used by *P. aeruginosa* for the initial catabolism of AGN.

MATERIALS AND METHODS

***P. aeruginosa* strains and media.** The *P. aeruginosa* strains used in this study are listed in Table 1. A minimal salts medium (8) containing 0.2% (wt/vol) glucose and 0.1% (wt/vol) $(NH_4)_2SO_4$ was used for routine growth protocols. When carbon source and/or nitrogen source studies were carried out, $(NH_4)_2SO_4$ and/or glucose were substituted as indicated in Results.

Growth curves. Culture turbidities in growth studies were determined with a Bausch & Lomb Spectronic 21 by monitoring A_{600}. Exponentially growing cul-

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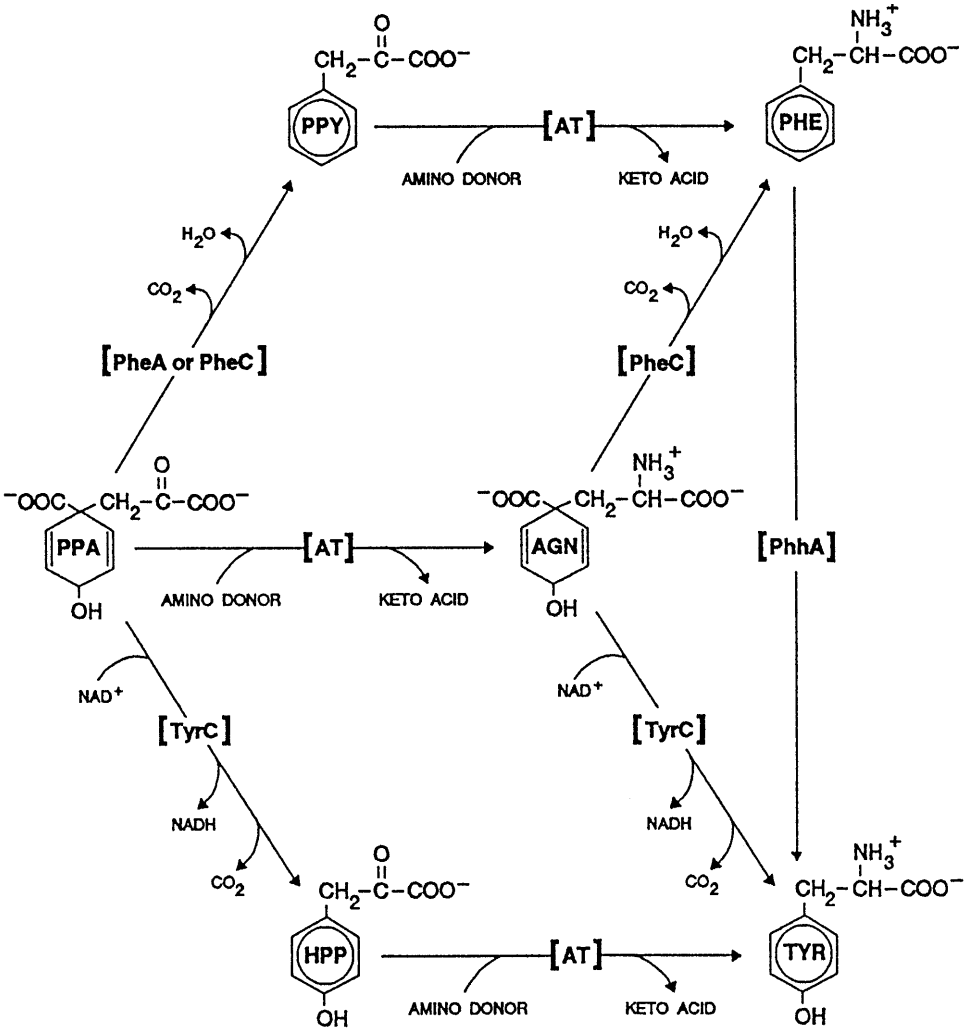

FIG. 1. Postprephenate flow routes to L-phenylalanine and L-tyrosine in *P. aeruginosa*. PheC (cyclohexadienyl dehydratase) and TyrC (cyclohexadienyl dehydrogenase) are broad-specificity enzymes. Thus, PheC can catalyze the prephenate dehydratase reaction (upper left) or the arogenate dehydratase reaction (upper right), whereas TyrC can catalyze the prephenate dehydrogenase reaction (lower left) or the arogenate dehydrogenase reaction (lower right). Multiple aromatic aminotransferases (AT) have been characterized (26), one of which is located in the periplasm (10). PheC is also located in the periplasm (30). HPP, 4-hydroxyphenylpyruvate; PHE, L-phenylalanine; PPA, prephenate; PPY, phenylpyruvate; TYR, L-tyrosine; PheA, bifunctional P protein; PhhA, phenylalanine hydroxylase.

tures were washed twice in minimal salts base (lacking a carbon or nitrogen source) and used to inoculate 5 ml of the desired medium contained in a 250-ml sidearm flask. These cultures were grown with vigorous shaking (270 rpm) in a gyratory shaker at 30°C.

AGN concentration. Culture supernatants were obtained (by microcentrifugation) from 50-μl samples removed from the growing cultures at the time points indicated in Results. The AGN concentration in the culture supernatants was then determined by employing *o*-phthalaldehyde derivatization and high-performance liquid chromatography as described by Bonner and Jensen (5).

TABLE 1. *P. aeruginosa* strains used in this study

Strain	Description[a]	Reference
PAO1	Prototroph	11
JS101	PAO1 *phhA* mutant, Hg^r	23
JS102	PAO1 *phhR* mutant, Hg^r	23
JS104	PAO1 *phhC* mutant, Hg^r	24
JG007	PAO1 *pheC* mutant, Hg^r	10
PA103	Prototroph	25
PA103-NG	PA103 *rpoN* mutant, Gm^r	25

[a] Hg^r, mercury resistant; Gm^r, gentamicin resistant.

Swarm plates and chemotaxis assays. Swarm plate assays were carried out at 30°C as described by Kamoun and Kado (16) employing semisolid (0.3%) Noble Agar (Difco Laboratories) plates made with chemotaxis buffer (20) and AGN supplied at a 1 mM final concentration.

The preparation of cells and the capillary tube assay of Adler (1) were performed at 30°C as described by Moulton and Montie (20), except that the chemotaxis buffer was adjusted to pH 7.2 and 1.5-ml microcentrifuge tubes were used (13) instead of the glass slide chamber. The terms response, threshold, peak, and background are used in this report as defined by Adler (1). Relative response is defined as the number of cells accumulating in a capillary tube containing a given nutrient divided by the background cell number. Chemotaxis competition experiments were carried out as described by Mesibov and Adler (18).

Biochemicals. AGN (99% pure with <0.1% L-phenylalanine), prepared from a *Salmonella typhimurium tyrA19* mutant (4) by the method of Zamir et al. (29), was utilized in the chemotactic and metabolic studies. L-Arginine (98% pure), L-phenylalanine (98% pure), disodium L-tyrosine (98% pure), and monopotassium L-glutamate (99 to 100% pure) were purchased from Sigma Chemical Company.

RESULTS

Identification of AGN as a nutrient and chemoattractant. Although the question is of obvious importance, nutritional

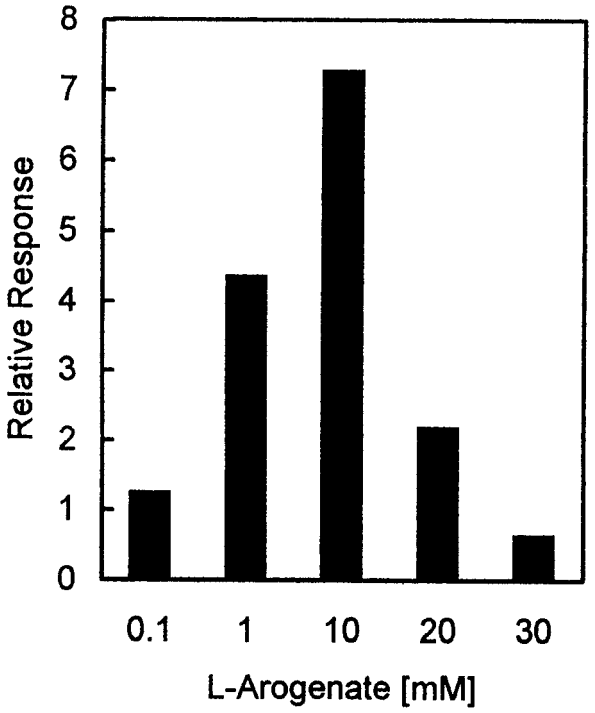

FIG. 2. Chemotaxis toward AGN by strain PAO1.

studies to determine whether *P. aeruginosa* or any other bacterium can catabolize AGN have not been done. Undoubtedly, this can be attributed to the general unavailability and expense of AGN. Soft-agar swarm plates are useful for the qualitative recognition of chemotaxis elicited by modest biochemical concentrations when the bacterium can metabolize the nutrient. Growth of the culture on semisolid agar in swarm assays results in the formation of a gradient as the nutrient is metabolized. Chemotactically competent cells respond to the gradient by moving outward from the point of inoculation while a fuzzy, slowly moving ring expanding to an area only slightly larger than that of the inoculum reflects random movement of motile cells that are nonchemotactic to the nutrient provided. The former result was realized when plates containing 1 mM AGN were inoculated with *P. aeruginosa* PAO1 (data not shown).

AGN, a unique chemoattractant. The concentration-dependent chemotactic response toward AGN in capillaries shown in Fig. 2 resembles those demonstrated for other amino acids with *P. aeruginosa* (6, 19, 20). Thus, chemotaxis increased with concentration to 10 mM AGN before steadily declining to levels less than the background. The peak relative response of 7.27, with a standard deviation of ±0.72, was the mean of three independent determinations. The average background value in capillaries containing only chemotaxis buffer was 4.8×10^3 cells.

A comparison of the chemotactic responses of amino acids that are structurally similar (L-tyrosine, L-phenylalanine, and L-glutamate) to AGN is given in Table 2. (AGN can be considered an analog of L-glutamate when the substituent groups at the C-1 ring position of AGN are viewed from a perspective across the top of the molecule.) L-Arginine, which produces the strongest amino acid response in *P. aeruginosa* (6), was also included in the study. The chemotactic threshold concentration of AGN ranged from 5-fold to 100-fold greater than the threshold values determined for L-phenylalanine and L-arginine, respectively. The peak response concentrations of AGN, L-phenylalanine, and L-arginine were each determined to be 10^{-2} M, while those of L-tyrosine and L-glutamate were 10-fold and 100-fold lower, respectively. The relative peak response to AGN was equivalent to those to L-phenylalanine and L-tyrosine but only 40 and 60% as great as those to L-arginine and L-glutamate, respectively.

TABLE 2. Comparison of chemotactic responses[a] of strain PAO1 toward AGN and selected amino acids in capillaries

Amino acid	Threshold concn (M)	Peak response concn (M)	Relative peak response
AGN	5×10^{-5}	10^{-2}	7.27
L-Arginine	5×10^{-7}	10^{-2}	18.62
L-Phenylalanine	1×10^{-5}	10^{-2}	7.41
L-Tyrosine	5×10^{-6}	10^{-3}	7.58
L-Glutamate	1×10^{-6}	10^{-4}	12.58

[a] The values shown are means of three determinations.

Experiments to assess whether L-tyrosine, L-phenylalanine, or L-glutamate could inhibit taxis to AGN were carried out to determine if the chemotactic response towards AGN might operate through a common chemoreceptor. In addition to the AGN provided in the capillary tube, the second amino acid was present in both the capillary tube and the cell suspension. A range of concentrations of AGN and compounds under test as possible inhibitors, including both intermediate and peak response concentrations, were evaluated. No inhibition of the chemotactic response for AGN occurred under any of the conditions tested when either L-tyrosine, L-phenylalanine, or L-glutamate was present as the second chemoattractant (data not shown). The apparent specificity demonstrated by AGN as a chemoattractant pointed to the importance of understanding the catabolism of this metabolite in nature.

Growth of strain PAO1 with AGN. *P. aeruginosa* PAO1 utilized AGN supplied in liquid medium as an excellent source of carbon or as a relatively poor source of nitrogen (Table 3). The doubling time (t_d) at 30°C on AGN as the sole source of carbon (2.4 h) was about the same as that on glucose or L-tyrosine and substantially less than that on fructose (4.0 h). Inoculum cell populations in exponential-phase growth on glucose-ammonia medium exhibited a rather lengthy lag phase when transferred to fresh medium containing 1 mM AGN as the sole source of carbon or nitrogen. Curiously, this lag phase was significantly extended when AGN was provided as the sole source of both carbon and nitrogen.

In experiments in which the utilization of AGN was monitored by high-performance liquid chromatographic analysis of the culture medium, it was observed that growth ceased at a time when only about 55% of the AGN had been utilized from the medium (Fig. 3). However, the remaining AGN was progressively depleted from the medium with continued incubation.

TABLE 3. Growth characteristics of strain PAO1 utilizing AGN as the sole source of carbon and/or nitrogen

Nutrient(s) derived from AGN[a]	Lag time (h)	t_d[b] (h)
Carbon	2–3	2.4 ± 0.2
Nitrogen	2–3	15.1 ± 0.7
Carbon + nitrogen	6–8	14.6 ± 0.6

[a] AGN was added to the medium to yield a final concentration of 1 mM.
[b] t_ds at 30°C were calculated from three or more independent growth curves.

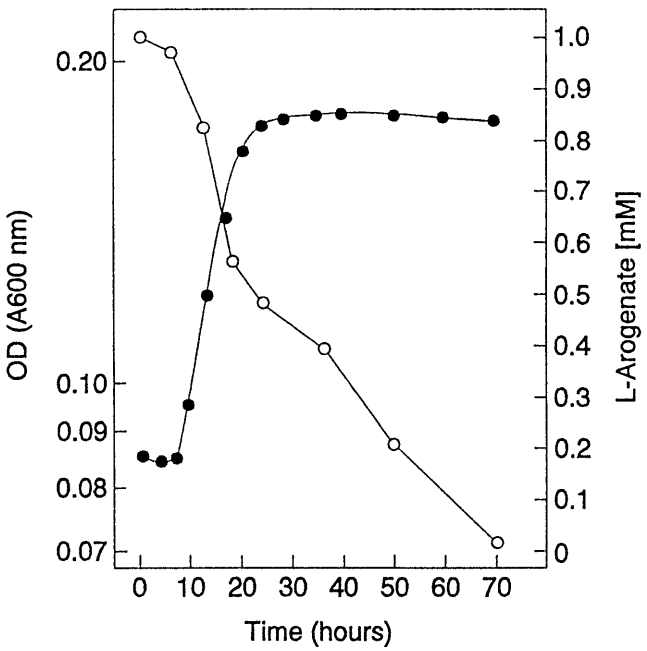

FIG. 3. Metabolism of AGN by strain PAO1 reflected by the growth pattern and depletion of 1 mM AGN. Symbols: ●, optical density (OD) at 600 nm; ○, AGN concentration in the medium.

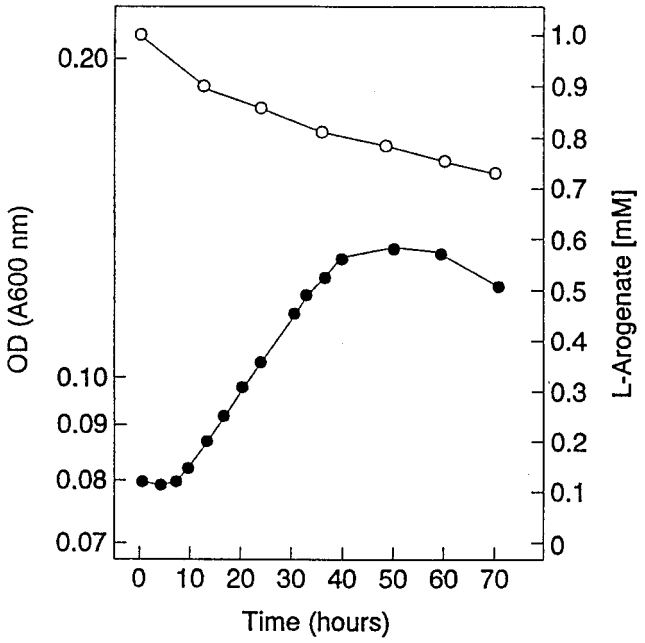

FIG. 4. Use of AGN as a sole source of nitrogen by strain JG007 reflected by the growth pattern and depletion of 1 mM AGN. Symbols: ●, optical density (OD) at 600 nm; ○, AGN concentration in the medium.

Key genes of AGN catabolism. *P. aeruginosa* mutants unable either to convert AGN directly to L-phenylalanine or to catabolize L-phenylalanine were characterized with respect to the ability to utilize AGN as a sole source of carbon or nitrogen (Table 4). Mutants carrying inactivated genes encoding PheC, PhhA, PhhC, or PhhR were unaffected in the ability to use AGN as a carbon source but exhibited markedly lower growth rates on AGN as a nitrogen source. A *phhR* regulatory-gene mutant fails to make a positively acting activator needed for transcription of *phhA* (23). The *phhR* mutant probably grows faster than the *phhA* mutant because of the leaky expression of *phh* operon genes in the absence of PhhR.

The limited utilization of AGN as a source of nitrogen by strain JG007 is shown in Fig. 4. This mutant exhibited a decreased growth yield that correlated with decreased (about 25%) utilization of AGN from the medium. When growth rates were limited with AGN as a source of nitrogen, a comparable inability to reach the growth yields typical of the parent strain was also observed with each of the *phh* operon mutants.

An *rpoN* mutant (PA103-NG) was not only severely limited in the ability to use AGN as a nitrogen source, but among all of the mutants characterized it was unique in that its utilization of AGN for carbon was negligible (t_d = 120 h).

TABLE 4. Ability of mutant strains of *P. aeruginosa* to utilize AGN[a] as a sole source of carbon or nitrogen

Mutant strain	Deficiency	Relative t_d[b] with AGN as source of:	
		C	N
JG007	Cyclohexadienyl dehydratase (PheC)	1.0	2.9
JS101	Phenylalanine hydroxylase (PhhA)	1.0	2.7
JS102	PhhR enhancer-binding protein (PhhR)	1.0	2.2
JS104	Aromatic aminotransferase (PhhC)	0.9	2.5
PA103-NG	σ^{54} transcription factor (RpoN)	30.0[c]	7.4

[a] AGN was added to the medium to yield a final concentration of 1 mM.

[b] t_ds were calculated by dividing the t_ds of the mutants by those of the parents. A relative value of 1 equals 2.4 h (AGN used for C) and 15.1 h (AGN used for N) for PAO1 (parent of the first four mutants) and 21 h (AGN used for N) for PA103 (parent of PA103-NG).

[c] This strain is a leaky L-glutamine auxotroph. The t_d on glucose-ammonia medium (4.0 h) was used for ratio calculations rather than the t_d of parent strain PA103.

DISCUSSION

Alternative pathways of AGN catabolism. Figure 5 illustrates the enzyme systems present in *P. aeruginosa* which might be linked with the catabolism of AGN. The periplasmic compartment possesses three enzymes which are able to carry out the overall conversion of chorismate to L-phenylalanine. PheC is located in the periplasm of *P. aeruginosa* (30), where it coexists (Fig. 5) with a species of chorismate mutase (AroQ) and aromatic aminotransferase (AT_p). The periplasmic location of AroQ has been demonstrated (10) with data similar to that shown by Xia et al. for *Erwinia herbicola* (28), and the presence of a periplasmic species of aromatic aminotransferase has also been demonstrated (10). This suite of enzymes in the spatial location of the periplasm constitutes the "hidden overflow pathway to L-phenylalanine" reported in 1983 (9). A given AGN molecule in the periplasm could be transformed to L-phenylalanine by PheC or to prephenate via periplasmic aminotransferase. A second possibility for transformation of AGN to prephenate is via oxidative deamination catalyzed by a membrane-associated oxidase. Cultures of *P. aeruginosa* can grow slowly (more than an order of magnitude more slowly than on AGN) on exogenous prephenate (7). The limiting step for prephenate metabolism is presumed to be transport into the periplasm. Any prephenate or AGN molecules which enter the cytoplasm are possible alternative substrates for TyrC (27), producing 4-hydroxyphenylpyruvate or L-tyrosine, respectively. L-Phenylalanine formed from AGN via PheC in the periplasm

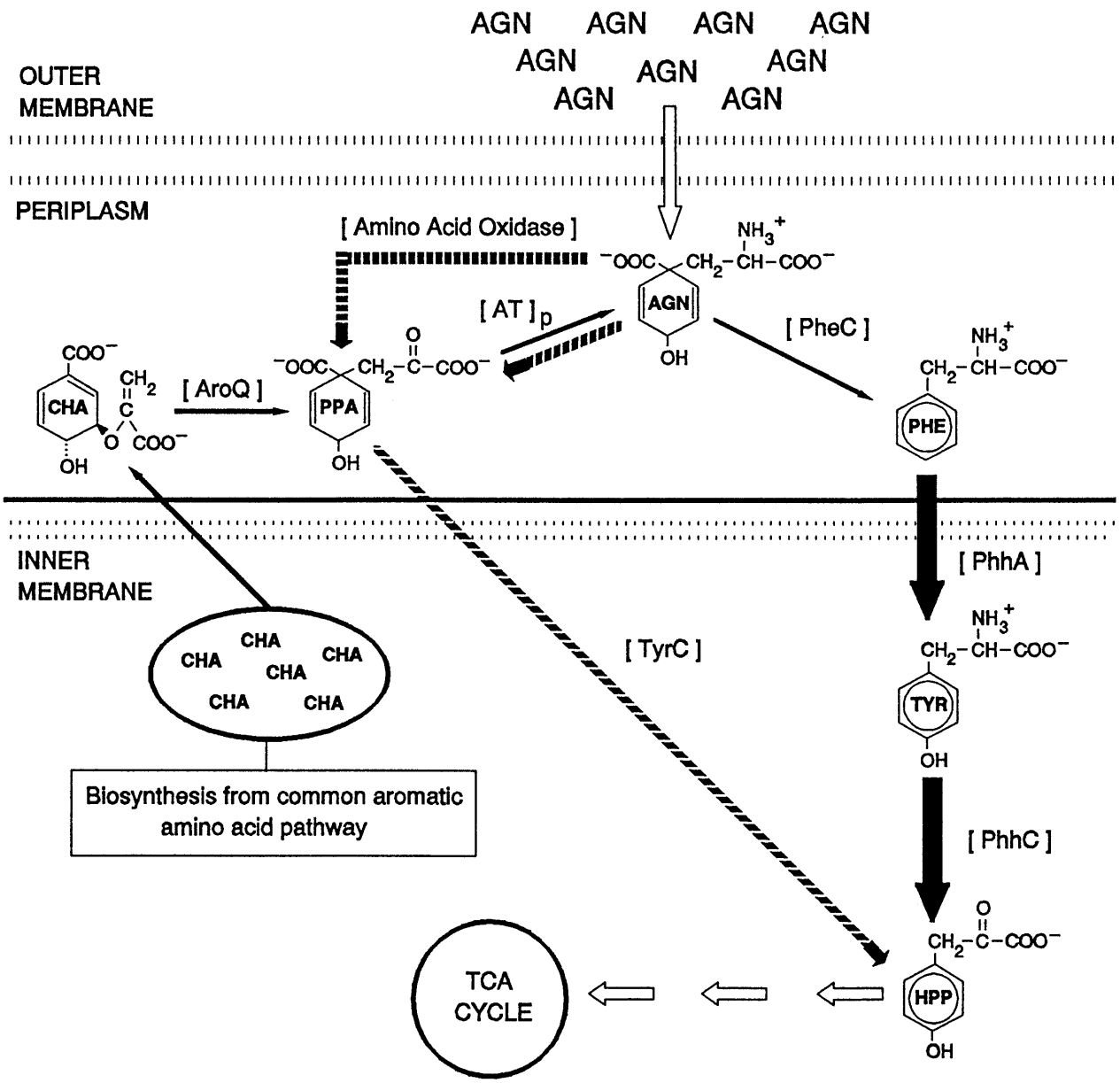

FIG. 5. Proposed pathway of initial catabolism of AGN. Solid, thin arrows show the three periplasmic enzymes of L-phenylalanine biosynthesis. Solid, thick arrows show steps catalyzed by enzymes of the *phh* operon. Striped lines show steps proposed to be the major flow route for AGN catabolism. CHA, chorismate; HPP, 4-hydroxyphenylpyruvate; PHE, L-phenylalanine; PPA, prephenate; TYR, L-tyrosine; AroQ, monofunctional chorismate mutase; PheC, cyclohexadienyl dehydratase; PhhA, phenylalanine hydroxylase; PhhC, aromatic aminotransferase; TyrC, cyclohexadienyl dehydrogenase; TCA, tricarboxylic acid.

would be expected to induce the *phh* operon, since PhhA and PhhC are known to be induced by exogenous L-phenylalanine (23). This provides the potential for overall conversion of AGN to 4-hydroxyphenylpyruvate. We have indeed found by Western blot analysis (24) that cultures catabolizing AGN exhibit maximally induced levels of PhhA, whereas *pheC* null mutants fail to produce induced levels of PhhA under the same growth conditions. PhhA is readily obtained as a soluble enzyme in *P. aeruginosa*, but it seems likely that it is membrane associated in vivo in view of its utilization of molecular oxygen for catalysis and the reducing environment present in the cytoplasm. The two catabolic pathways shown in Fig. 5 converge at 4-hydroxyphenylpyruvate, the product of the initial step of L-tyrosine catabolism. Subsequent steps prior to ring cleavage include formation of 4-hydroxyphenylacetate and 3,4-dihydroxyphenylacetate.

L-Phenylalanine is an extremely poor source of carbon (t_d, 25.5 h), whereas L-tyrosine is an excellent carbon source (t_d, 2.4 h). This is surprising because phenylalanine-inducible PhhA converts L-phenylalanine to L-tyrosine. PhhA is a low-abundance protein and presumably is the rate-limiting step for phenylalanine catabolism. Since the growth rate on AGN is equal to that on L-tyrosine, it seems likely that AGN catabolism largely bypasses L-phenylalanine as an essential intermediate. This could occur via utilization of AGN or prephenate by TyrC. However, direct utilization of AGN by TyrC acting in a catabolic mode has been ruled out for two reasons. Firstly, L-tyrosine (the product of AGN utilization) is a potent feed-

back inhibitor of TyrC (27). Secondly, the PhhC-deficient mutant cannot grow on L-tyrosine as a carbon source. Since this mutant is competent for normal growth on AGN as a carbon source, a catabolic pathway for AGN must be available which bypasses the PhhC step. Transformation of AGN to prephenate and utilization of prephenate by TyrC bypasses L-tyrosine as an intermediate step, thus avoiding the restraint otherwise imposed on TyrC by feedback inhibition. Additionally, this pathway would be PhhC independent. The proposed flux of carbon through the prephenate pathway shown in Fig. 5 is also consistent with the observation that inactivation of PheC, PhhA, and PhhC does not affect growth rates on AGN as a sole source of carbon. Confirmation of this pathway awaits the isolation of a mutant deficient in *tyrC*.

AGN as a sole source of nitrogen. When AGN was presented to *P. aeruginosa* cultures as the only source of both carbon and nitrogen, the nitrogen metabolism was clearly limiting to growth. When flux through the PheC-PhhA-PhhC pathway was blocked by mutations, ability to use AGN as a nitrogen source was decreased. We conclude that the TyrC pathway of Fig. 5 is a high-flux pathway of AGN catabolism which intercepts the pathway of tyrosine catabolism at the level of 4-hydroxyphenylpyruvate. A simultaneously present low-flux (PheC-PhhA-PhhC) pathway is not needed to sustain maximal growth rates on AGN as a carbon source but is needed for maximal growth rates on AGN as a nitrogen source. To use amino acids as a nitrogen source, ammonia must be generated. This could be provided directly by oxidative deamination of AGN to produce prephenate and ammonia. In *P. aeruginosa*, most amino acids have been shown to be subject to oxidative deamination under conditions of nitrogen limitation (17). Production of L-glutamate by transamination catalyzed by periplasmic aromatic aminotransferase or by PhhC could generate ammonia indirectly, e.g., via coupling with aspartase or glutamate dehydrogenase.

A toxic intermediate? L-Phenylalanine is a poor source of carbon. This is curious, since L-phenylalanine induces phenylalanine hydroxylase, which produces L-tyrosine, an extremely good source of carbon. It appears that provision of L-phenylalanine results not only in the production of L-tyrosine but in the production of a toxic intermediate as well. A good candidate is phenylacetate, which is highly inhibitory to *P. aeruginosa* cultures growing on L-tyrosine as a carbon source (24). Is it possible that phenylacetate interferes with 4-hydroxyphenylacetate as a substrate for the monooxygenase step of L-tyrosine catabolism?

When AGN is utilized for growth, the culture stops growing at a time when almost half of the AGN is still available in the medium. It may be that inhibition of L-tyrosine catabolism by an L-phenylalanine catabolite also occurs under these conditions. Since this phenomenon was also observed with *pheC* and *phh* operon mutants, the toxic intermediate must be generated through another pathway. Perhaps a fraction of the prephenate formed by deamination or transamination of AGN could be utilized by intracellular prephenate dehydratase (PheA) to produce phenylpyruvate (and hence phenylacetate).

Chemotaxis to AGN. The capacity to detect nutrients in the environment and catabolize them facilitates bacterial growth and survival under a broad spectrum of ambient conditions. Chemotaxis is thought to be an important process for gram-negative, free-living bacteria. *P. aeruginosa* was an ideal organism for initial studies with AGN, since the conditions for chemotaxis in this bacterium are well defined (20) and because it has been a focal point of studies keyed to the relationship of AGN and aromatic metabolism. The taxis response pattern of *P. aeruginosa* to AGN and the lack of taxis inhibition by structurally similar amino acids indicated that AGN was unique among the test group of amino acids. Although taxis inhibition can be explained by causes other than competition for binding sites (18, 21), lack of inhibition is strong evidence that two chemoattractants bind to different chemoreceptors. This is noteworthy, since L-phenylalanine, which is the facile aromatization product of AGN under acidic conditions (29), produced a response profile that was most similar to that produced by AGN. The competition experiments rule out the possibility that apparent taxis to AGN is actually due to taxis to L-phenylalanine generated nonenzymatically from AGN.

Chemotaxis is known to be advantageous to bacteria in nutrient-poor environments. An organism that can locomote toward a desirable nutrient would have an increased chance of survival. An intriguing possibility is that plant-associated saprophytes and pathogens may utilize available molecules of AGN, not only as a source of metabolically expensive nutrients but also to undermine the production by plants of antimicrobial phytoalexins which are derived from L-phenylalanine. In higher plants, L-phenylalanine appears to be derived exclusively from AGN (12). It would be interesting to know whether phytopathogenic pseudomonad groupings that are known to possess AGN-utilizing enzymes, e.g., *P. syringae* or *Xanthomonas campestris* (15), rely upon taxis to AGN and subsequent utilization as pathogenicity determinants.

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