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Genetic and Physiological Characterization of *Pseudomonas aeruginosa* Mutants Affected in the Catabolic Ornithine Carbamoyltransferase

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In *Pseudomonas aeruginosa* arginine can be degraded by the arginine "dihydrolase" system, consisting of arginine deiminase, catabolic ornithine carbamoyltransferase, and carbamate kinase. Mutants of *P. aeruginosa* strain PAO affected in the structural gene (*arcB*) of the catabolic ornithine carbamoyltransferase were isolated. First, an *argF* mutation (i.e., a block in the anabolic ornithine carbamoyltransferase) was suppressed specifically by a mutationally altered catabolic ornithine carbamoyltransferase capable of functioning in the anabolic direction. The suppressor locus *arcB*(Su) was mapped by transduction between *hisII* and *argA*. Second, mutants having lost suppressor activity were obtained. The Su^- mutations were very closely linked to *arcB*(Su) and caused strongly reduced ornithine carbamoyltransferase activities in vitro. Under aerobic conditions, a mutant (PAO630) which had less than 1% of the wild-type catabolic ornithine carbamoyltransferase activity grew on arginine as the only carbon and nitrogen source, at the wild-type growth rate. When oxygen was limiting, strain PAO630 grown on arginine excreted citrulline in the stationary growth phase. These observations suggest that during aerobic growth arginine is not degraded exclusively via the dihydrolase pathway.

Pseudomonas aeruginosa has the ability to utilize arginine as the sole carbon and nitrogen source in minimal media. The rapid degradation of arginine is a taxonomic characteristic of fluorescent pseudomonads (15, 20). The "arginine dihydrolase" pathway converts arginine to ornithine, ammonia, and bicarbonate and generates ATP from ADP and inorganic phosphate (16). Three enzymes involved in this pathway have been demonstrated in cell extracts of *P. aeruginosa*: arginine deiminase (EC 3.5.3.6) degrades arginine to citrulline (6), and a catabolic ornithine carbamoyltransferase (EC 2.1.3.3), coupled with carbamate kinase (EC 2.7.2.2), catalyzes the phosphorolysis of citrulline (18, 19). The reaction, ornithine + carbamoylphosphate $\rightleftharpoons$ citrulline + P_i, is of particular interest since it is catalyzed by two structurally different ornithine carbamoyltransferases. An anabolic enzyme coded by the *argF* locus in *P. aeruginosa* (4) is active in the biosynthesis of citrulline. In vitro, this enzyme is inefficient in the reverse reaction because citrulline forms a dead-end complex with the enzyme and is a very poor substrate (17). The catabolic ornithine carbamoyltransferase is active in the phosphorolysis of citrulline but has little biosynthetic activity in vitro owing to a strongly cooperative saturation function and a high half-saturation concentration for carbamoylphosphate (19).

In vivo, the arginine dihydrolase enzymes of *Pseudomonas* species are most active when oxygen is limiting (8, 20). However, the precise function of the dihydrolase pathway is difficult to assess, because no mutants blocked in this pathway have been described. It is possible to isolate mutants of *P. aeruginosa* that are unable or barely able to grow on arginine as the only carbon source; however, none of these mutants has been found to be defective in the dihydrolase pathway (our unpublished data).

We have now isolated mutants of *P. aeruginosa* strain PAO which have an altered catabolic ornithine carbamoyltransferase with greatly reduced or undetectable activity, and we have mapped a locus, *arcB* (for arginine catabolism; reference 1), specifying this enzyme. The properties of our *arcB* mutants suggest that the

utilization of arginine by *P. aeruginosa* does not depend solely on the dihydrolase pathway.

MATERIALS AND METHODS

Bacterial strains. All mutants were derived from *P. aeruginosa* PAO and are shown in Table 1.

Media. Nutrient yeast broth, nutrient agar, minimal medium, and medium P have been described (4, 21). The concentration of amino acids was 1 mM when used as supplement and 20 mM when used as the sole carbon and nitrogen source in medium P.

Isolation of mutants. (i) Arg^+ derivatives from strain PAO522 were obtained by plating approximately 10^8 exponentially growing cells on minimal medium with adenine. A few crystals of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) were placed in the center of the plate. After incubation at 37°C for 48 h, single colonies growing within a 5-cm radius around the mutagen were picked and purified on the selective medium. (ii) Arg derivatives of strain PAO524 were obtained by mutagenesis with ethyl methane sulfonate in nutrient yeast broth (26) or with NG (50 μg/ml) in 0.1 M citrate buffer (pH 5.6; exposure 10 min at 37°C without aeration), followed by carbenicillin enrichment (27).

TABLE 1. *Strains of P. aeruginosa*

Strain	Genotype	Origin, method of construction, or reference
PAO1	Prototroph, *cml-2*	(5)
PAO18	*pro-64 pur-66*	(4)
PAO315	*argG9*	(4)
PAO504	*met-9011 ilv-200*	This study; the *met-9011* marker (10) was isolated by H. Matsumoto
PAO522	*pur-136 argF2*	(4)
PAO524	*pur-136 argF2 arcB1*(Su)	NG-induced Arg^+ derivative of PAO522
PAO527	*ilv-226 his-4 lys-12 met-28 trp-6 pro-82 argF2*	Leu^+ Arg recombinant of PAO522(R68.45) × PAO227 (27)
PAO529	*pur-136 argF2 arcB1*(Su) *his-16*	*hisII* derivative of PAO524
PAO531	*his-35 argF10*	Pur^+ Arg recombinant of PAO25(R68.45) × GMA253 (2, 11)
PAO532	*his-35 argF2*	Pur^+ His recombinant of GMA153(FP2) × PAO522 (11)
PAO533	*his-35 argF3*	Pur^+ Arg recombinant of PAO21(R68.45) × GMA253 (4, 11)
PAO535	*ilv-226 his-4 argA127 met-28 argF2*	Derived from PAO222 (26) and PAO522 by R68.45-mediated crosses
PAO622	*pur-136 argF2 arcB2*	EMS[a]-induced Arg derivative of PAO524
PAO623	*argF2 arcB2*	Pur^+ transductant of G101c.PAO1 × PAO622
PAO624	*pur-136 argF2 arcB4*	EMS-induced Arg derivative of PAO524
PAO625	*argF2 arcB4*	Pur^+ transductant of G101c.PAO1 × PAO624
PAO627	*pur-136 argF2 arcB7*	NG-induced Arg derivative of PAO524
PAO628	*argF2 arcB7*	Pur^+ transductant of G101c.PAO1 × PAO627
PAO629	*pur-136 argF2 arcB9*	NG-induced Arg derivative of PAO524
PAO630	*argF2 arcB9*	Pur^+ transductant of G101c.PAO1 × PAO629

[a] EMS, Ethyl methane sulfonate.

Strain construction. Donor strains carrying the conjugative plasmids FP2 (4) or R68.45 (2) were isolated as described before. Strains with desired genotypes were constructed by R68.45 crosses (3) or G101c transductions (V. Krishnapillai, unpublished data).

Matings on the plate. Donor cells carrying FP2 or R68.45 were crossed with recipients as previously described (3, 21).

Transductions. The generalized transducing phage G101 was used in the standard procedure (4).

Assay of ornithine carbamoyltransferase in whole cells. Bacteria were grown in nutrient yeast broth to approximately 5×10^8 cells per ml (exponential phase) with good aeration at 37°C. Cells were centrifuged, suspended in ½ volume of 0.9% (wt/vol) NaCl, and permeabilized with toluene at a ratio of 0.1 ml of toluene per 2 ml of cell suspension. The mixture was incubated at 37°C for 15 min without shaking. Ornithine carbamoyltransferase activity was estimated at pH 6.2, 7.3, and 8.7. At pH 7.3, the catabolic ornithine carbamoyltransferase is optimally active, whereas the activity of the anabolic ornithine carbamoyltransferase is very low (19). At pH 8.7, the activity of the anabolic ornithine carbamoyltransferase is high, but that of the catabolic enzyme is negligible (19). The incubation mixtures contained, in a final volume of 1 ml: 0.4 ml of cell suspension, 0.1 M imidazole-hydrochloride buffer (pH 6.2), 10 mM carbamoylphosphate, and 10 mM L-ornithine; or: 0.1 ml of cell suspension, 0.1 M Tris-hydrochloride buffer (pH 7.3), 10 mM potassium phosphate (pH 7.3), 10 mM carbamoylphosphate, and 10 mM L-ornithine; or: 0.2 ml of cell suspension, 0.2 M Tris-hydrochloride buffer (pH 8.7), 10 mM carbamoylphosphate, and 10 mM L-ornithine. The reaction was terminated by the addition of 2 ml of color reagent (22). Blanks were obtained by adding ornithine after incubation. The samples were boiled for 20 min and allowed to cool in darkness. Enzyme activity was estimated by comparison of the color produced with that of a citrulline standard. This test was useful in a qualitative screening of ornithine carbamoyltransferase mutants.

Assay of ornithine carbamoyltransferase activity in cell extracts. Cells were grown in medium P with good aeration and harvested in the exponential phase. In some experiments, oxygen supply was limited. This was done by slowly shaking a hermetically sealed 1-liter flask containing 540 ml of medium P and an inoculum of 10^7 cells per ml. Cells were collected in the stationary phase (approximately 3×10^8 cells per ml) and suspended in 50 mM phosphate buffer (pH 7.5) (ca. 100 mg [wet weight] per ml of buffer). Cell extracts were prepared by sonication in a Mullard Sonic Oscillator at 4°C and 20 kilocycles per s for 5 min. The activity of the anabolic ornithine carbamoyltransferase was measured at pH 8.5 in 60 mM EDTA-10 mM carbamoylphosphate-10 mM L-ornithine. The catabolic ornithine carbamoyltransferase was assayed at pH 7.5 in 150 mM imidazole-hydro-

chloride buffer–10 mM carbamoylphosphate–10 mM L-ornithine–10 mM phosphate. Other assay conditions were as previously described (19). Arsenolysis of citrulline was determined by the method of Legrain and Stalon (7) in 100 mM arsenate (pH 6.8)–10 mM L-[*carbamoyl*-^{14}C]citrulline (50 μCi/mmol).

Citrulline determination. Excretion of citrulline by bacteria was detected in culture medium. After centrifugation of 4 ml of medium, citrulline was determined in the supernatant by the colorimetric method used for the ornithine carbamoyltransferase assay.

Protein determination. Protein concentrations were measured by the method of Lowry et al. (9).

Chemicals. Ethyl methane sulfonate was obtained from Eastman, NG came from K&K Laboratories, and carbamoylphosphate-Li_2 was obtained from Sigma. L-[*carbamoyl*-^{14}C]citrulline was obtained from the Radiochemical Centre, Amersham.

RESULTS

Isolation of an *argF* suppressor. Strain PAO522, a citrulline auxotroph, was chosen as the parent because the spontaneous reversion of the *argF2* allele to prototrophy was very rare (reversion frequency $<10^{-8}$). After NG mutagenesis of PAO522, Arg^+ colonies were selected. The Arg^+ phenotype could be due to different events: (i) mutation in *argF* (reversion); (ii) informational suppression, e.g. by nonsense or missense suppressors; (iii) suppression by a bypass reaction, notably by a modified catabolic ornithine carbamoyltransferase, as described for a mutant of *P. putida* IRC204 (19). A mutant of type (iii) was sought as follows. Eight Arg^+ colonies were purified and tested for ornithine carbamoyltransferase activity in whole cells. All clones had ornithine carbamoyltransferase activity at pH 7.3 (indicative of catabolic ornithine carbamoyltransferase) and at pH 8.7 (indicative of anabolic ornithine carbamoyltransferase), whereas strain PAO522 had no activity at pH 8.7 but wild-type activity at pH 7.3. The Arg^+ clone exhibiting the highest activity at pH 8.7 was named PAO524 and examined further.

The *argF* locus is cotransducible with *argG* in *P. aeruginosa* (4). (Fig. 1 shows a map of the chromosome.) Strain PAO524 was shown not to be a revertant by transductional analysis. When bacteriophage G101 was propagated on PAO524 and $argG^+$ transductants of PAO315 (*argG9*) were selected, 24% had inherited the *argF* allele. By comparison, a lysate of phage G101 grown on PAO522 gave 28% cotransduction between *argG* and *argF*. (In each case 400 transductants were analyzed.) Thus, the presence of a suppressor mutation was indicated in PAO524. This result was confirmed and extended by crosses with the conjugative plasmid R68.45. We have shown elsewhere (3) that R68.45 can be used like a large generalized transducing phage in *P. aeruginosa*. The *argG* marker is the only locus known to be cotransducible with *argF* by phage G101. By contrast, several markers are linked to *argG* in R68.45-mediated plate matings. Usually, markers located about 10 min apart on the chromosome give about 10% linkage in R68.45 matings (3). Table 2 summarizes the results of the R68.45 crosses. Linkage of *argF* to *met-9011*, *pur-66*, and *argG* was not significantly different when the suppressor strain PAO524(R68.45) was used instead of the parent PAO522(R68.45). Thus the suppressor locus was not situated in the vicinity of *argF* and seemed to lie outside the *pur-66* - *met-9011* region. As we show below, the suppressor activity is likely to be due to mutation in the structural gene for the catabolic

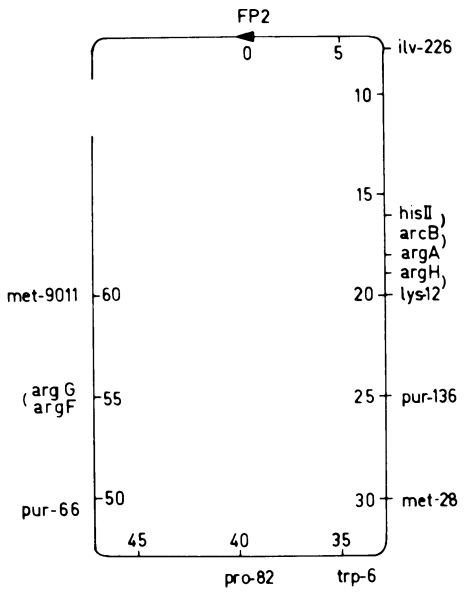

FIG. 1. *Chromosome map of P. aeruginosa PAO. The map is based on earlier results (4) and shows the positions of markers relevant to this study. his-4, his-35, and his-16 lie in the hisII region (11).*

TABLE 2. *Conjugational linkage of argF to markers in the late chromosome region*

Recipient strain[a]	Marker selected	Linkage of the unselected marker *argF* (%)	
		PAO522(R68.45)[b]	PAO524(R68.45)[b]
PAO504	*met-9011*$^+$	3	9
PAO18	*pur-66*$^+$	8	11
PAO315	*argG9*$^+$	76	81

[a] Matings were carried out on the plate. Two hundred recombinants of each class were scored.

[b] Donor strains.

ornithine carbamoyltransferase. Hence we call the suppressor locus *arcB1*(Su).

Mapping of *arcB1*(Su). Strain PAO524 was made male by infection with the sex factor FP2. An interrupted mating (4) with a nalidixic acid-resistant derivative of PAO522 as the recipient gave an approximate map location of *arcB1*(Su) at 20 min from the FP2 origin. Since this chromosome region contains a number of known closely linked markers (4), appropriate strains were constructed allowing mapping of *arcB1*(Su) by conjugation and transduction.

A three-factor cross (Table 3) established that *arcB1*(Su) lies between *his-4* (at 16 to 17 min) and *lys-12* (at 20 min). The high conjugational linkage of *arcB1*(Su) to *his-4* suggested that the two markers might be cotransducible. This was verified by G101 transduction: *arcB1*(Su) was linked to *his-4*, but not to *lys-12*. Selection for *arcB1*(Su) gave 21% linkage to *his-4*, whereas 3% linkage was obtained in the reciprocal cross. This phenomenon will be discussed later.

The *argA* locus is located between *his-4* and *lys*, but it is cotransducible with neither marker (4). Ten percent cotransduction was found between *arcB1*(Su) and *argA*. The donor was phage G101 grown on PAO524, the recipient was PAO535, and selection was made for *arcB*(Su) (Arg^+). We deduce the gene order *his-4–arcB–argA–lys* (Fig. 1).

***arcB1*(Su) suppresses *argF* specifically.**

TABLE 3. *Location of arcB1(Su) relative to his-4 and lys-12*

Marker selected[a]	No. of recombinants scored	Recombinant phenotype	Frequency (%)	Minimal number of crossovers required
his-4$^+$	200	His^+ Arg Lys	28.5	2
		His^+ Arg^+ Lys	48.5	2
		His^+ Arg^+ Lys^+	21	2
		His^+ Arg Lys^+	2	4
arcB1(Su)	196	His Arg^+ Lys	16.8	2
		His^+ Arg^+ Lys^+	36.2	2
		His^+ Arg^+ Lys	43.4	2
		His Arg^+ Lys^+	3.6	2
lys-12$^+$	200	His Arg Lys^+	45	2
		His^+ Arg Lys^+	45.5	2
		His Arg^+ Lys^+	8	2
		His^+ Arg Lys^+	1.5	4

[a] PAO527 and PAO524(FP2) were mated on the plate. Each marker was selected in turn; the two other markers were scored by replica plating onto appropriately supplemented media. When *lys-12* was the selected marker, the frequency of recombinants was low, probably because the counterselective marker in the donor (*pur-136*) is near *lys-12* (see Fig. 1). The Arg^+ phenotype of *arcB1*(Su) was used to select and score for this marker. The results are consistent with the gene order FP2-origin–*his-4–arcB–lys-12*.

If *arcB1*(Su) specifies a modified catabolic ornithine carbamoyltransferase, it should suppress any *argF* mutation. Three independently induced mutations (*argF2*, *argF3*, and *argF10*) have previously been shown to be very closely linked but distinct, because intragenic recombination between these alleles occurs at a low frequency (4). Strains carrying the same *his* mutation (*his-35*, which lies near *his-4*) but different *argF* alleles were used as recipients in transduction with phage G101 grown on PAO524 as the donor. In each case, *arcB1*(Su) suppressed the arginine auxotrophy and the suppressor locus was linked to *his* (Table 4).

The *arcB1*(Su) mutation appears to be unrelated to two known informational suppressors, *supB* and *sup-1*, previously described in *P. aeruginosa*. The *supB* mutation suppresses amber mutations (13). An amber mutant (*sus-1*) of bacteriophage PRD1 (which specifically attacks strains harboring incP-1 R plasmids) was able to grow in a *supB*(R^+) host, but not in an *arcB1*(Su) (R^+) strain. The *sup-1* locus suppresses several auxotrophic mutations and *sus* mutations in the virulent phage E79; *sup-1* is probably not of the amber or ochre type (26). E79 *sus-1*, a suppressor-sensitive mutant, could be propagated in a *sup-1* host, but not in the *arcB1*(Su) strain PAO524.

Thus, the genetic evidence indicates that the *arcB1*(Su) mutation specifically restores anabolic ornithine carbamoyltransferase activity. This could be done by *arcB1*(Su)-directed modification of mutant *argF* proteins or by modification of the catabolic ornithine carbamoyltransferase protein. The second possibility is more likely, as shown by the biochemical analysis of PAO524.

Properties of ornithine carbamoyltransferase in strain PAO524. Ornithine carbamoyltransferase activity of PAO524 was measured in cell extracts as a function of pH. Figure

TABLE 4. *Suppression of different argF alleles by arcB1(Su)*

Recipient[a]	Linkage *his-35–arcB1*(Su) (%)	
	Selection for His^+	Selection for Arg^+
PAO532 (*argF2*)	5.5	46.5
PAO533 (*argF3*)	3.0	47.0
PAO531 (*argF10*)	3.5	37.5

[a] In each cross, G101.PAO524 was the donor and His^+ and Arg^+ transductants were selected. Arg^+ transductants arising because of intragenic recombination in *argF* can be disregarded (4). Two hundred transductants of each class were scored for the coinheritance of the unselected marker.

2 shows that the pH optimum was 8.2. The shape of the pH optimum curve suggests that a single enzyme species was present, distinct from the situation in the wild type (PAO1), where the mixture of anabolic and catabolic ornithine carbamoyltransferases results in two pH optima (Fig. 2). In strain PAO522, the parent of PAO524, only the catabolic enzyme (pH optimum 7.3) was present.

The catabolic ornithine carbamoyltransferase in PAO1 showed a very strong cooperativity for carbamoylphosphate. A similar behavior has been described for the enzyme of *P. putida* (19). The addition of 25 mM phosphate as an activator abolished this allosteric behavior of the PAO1 catabolic ornithine carbamoyltransferase. In PAO524, the affinity for carbamoylphosphate was greatly enhanced; phosphate was not required for activation of the enzyme, but caused slight product inhibition (data not shown). The

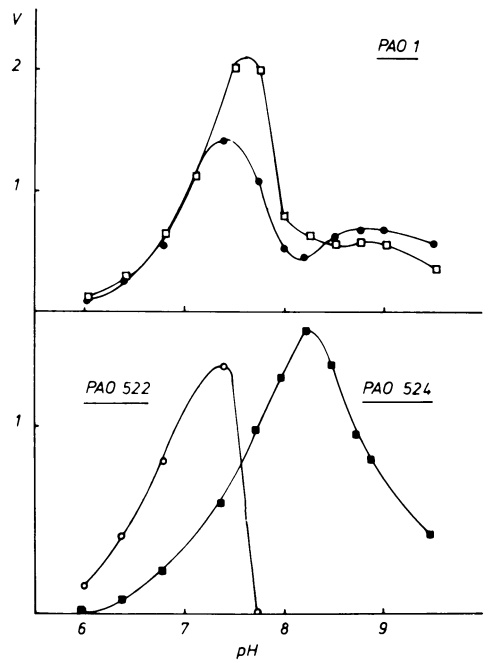

FIG. 2. *Formation of citrulline as a function of pH in cell extracts of PAO1, PAO522, and PAO524. Ornithine carbamoyltransferase activity (v) is expressed as micromoles of citrulline formed per 15 min in a reaction mixture containing 60 mM EDTA buffer, 3 mM* L*-ornithine, and 10 mM carbamoylphosphate. Cell extracts were obtained from cells grown aerobically in minimal medium and harvested in the exponential phase. Each tube contained 100 μg of protein.* (●) *PAO1 extract without phosphate;* (□) *PAO1 extract assayed in the presence of 25 mM phosphate;* (○) *PAO522 extract without phosphate;* (■) *PAO524 extract without phosphate.*

apparent molecular weight of the anabolic ornithine carbamoyltransferase is ca. 110,000, and that of the catabolic ornithine carbamoyltransferase is ca. 420,000, as determined by gel filtration (8). When a crude extract of PAO524 was chromatographed on Sephadex G-200, a single symmetrical peak of ornithine carbamoyltransferase activity was found at the position characteristic of the catabolic enzyme (data not given).

The ornithine carbamoyltransferase of PAO524 was weakly induced by arginine (as the only carbon and nitrogen source) and strongly induced by poor aeration (in the stationary phase). This regulation pattern is typical of the catabolic ornithine carbamoyltransferase (8) and arginine deiminase. The anabolic enzyme, by contrast, is repressed by arginine (25), and aeration has little effect on the specific activity.

These results indicate that the *arcB1* (Su) locus modifies some catalytic properties of the catabolic ornithine carbamoyltransferase.

Isolation of suppressor-negative derivatives. Strain PAO524 was mutagenized with ethyl methane sulfonate or NG, and Su^- derivatives (citrulline auxotrophs) were isolated. Pur^+ transductants of the four Su^- strains were selected and used in further physiological experiments to eliminate the possible interference of the purine requirement.

Growth characteristics of Su^- strains. Under aerobic conditions, PAO1 had a doubling time of ca. 75 min in liquid medium P with 20 mM L-arginine as the sole carbon and nitrogen source. The same doubling time was found for PAO524 [*arcB1* (Su)] and its four Su^- derivatives. All strains could utilize citrulline as the nitrogen source in the presence of glucose with equal efficiency. Neither the wild type nor the mutants could grow on citrulline as the only carbon and nitrogen source. Under conditions of poor aeration (described in Materials and Methods), all strains entered the stationary phase at a cell concentration of 3×10^8 to 4×10^8 cells per ml in medium P plus 20 mM L-arginine. Although under these conditions the one Su^- strain that had no detectable ornithine carbamoyltransferase activity (PAO630) grew somewhat more slowly (doubling time ca. 120 min) than the other strains (doubling time ca. 85 min), this growth differential was small and did not result in an arginine utilization-negative phenotype.

Properties of ornithine carbamoyltransferase in Su^- strains. Crude extracts were prepared from cells grown to stationary phase with poor aeration to allow maximal formation of catabolic ornithine carbamoyltransferase. En-

zyme activity was assayed in the direction of citrulline synthesis and citrulline degradation (arsenolysis). In one strain, PAO630, no citrulline-synthesizing activity could be found at any pH; a very weak arsenolytic activity (less than 1% of the wild-type level) was detectable. Whether this residual activity is due to catabolic ornithine carbamoyltransferase or another enzyme is not known. In three other strains the ornithine carbamoyltransferase activity was very low but clearly detectable (Table 5). The same result was obtained when ornithine carbamoyltransferase activity was estimated in toluenized whole cells. Figure 3 shows that in each Su^- strain the pH optimum of the ornithine carbamoyltransferase was shifted to a lower pH by comparison with the Su^+ enzyme of PAO524 (Fig. 2). Furthermore, the ornithine carbamoyltransferases of PAO625 and PAO628 resembled the wild type in that inorganic phosphate was required for activation. By contrast, the low activity in PAO623 could not be stimulated by phosphate.

Citrulline excretion. Loss of catabolic ornithine carbamoyltransferase activity in vivo should lead to citrulline excretion under conditions of induction. This prediction was verified in Table 5. When arginine, used as the carbon and nitrogen source, was exhausted in the growth medium of poorly aerated cultures, accumulation of citrulline in the medium was noted during the stationary growth phase for three *arcB*(Su^-) mutants, but not for the wild-type PAO1 or the *arcB1*(Su) mutant PAO524. When the aeration was good, less than 5% of the arginine was excreted as citrulline by both PAO1 and PAO630.

Genetic analysis of Su^- derivatives. The mutations causing the Su^- phenotype were an-

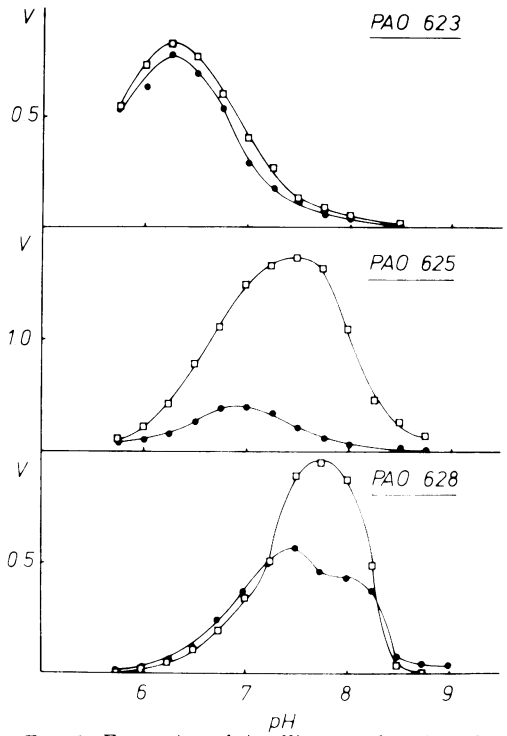

FIG. 3. *Formation of citrulline as a function of pH in cell extracts of Su^- strains. Ornithine carbamoyltransferase activity (v) is expressed as micromoles of citrulline formed per 20 min in a reaction mixture containing 60 mM EDTA buffer, 3 mM* L-*ornithine, and 20 mM carbamoylphosphate. Cell extracts were obtained from cells grown in medium P + 20 mM* L-*arginine under O_2 limitation and harvested in the stationary phase. Each tube contained 41 μg (PAO623), 16 μg (PAO625), or 72 μg of protein (PAO628).* (●) *Incubation without phosphate;* (□) *incubation in the presence of 25 mM inorganic phosphate.*

TABLE 5. *Ornithine carbamoyltransferase activity and citrulline excretion in arcB strains*

Strain	Time in stationary phase[a] (h)	Ornithine carbamoyltransferase sp act[b]		Citrulline excretion[c] (μmol/ml)	Residual arginine[c] (μmol/ml)
		Synthesis of citrulline	Arsenolysis of citrulline		
PAO1	15	2218 (pH 7.5)	79	2.5	<0.1
PAO524	15	3334 (pH 8.2)	88	2.35	<0.1
PAO623	15	30 (pH 6.2)	ND	3.3	10.8
	60	58	1.6	14.3	<0.1
PAO625	15	326 (pH 7.5)	1.7	2.7	<0.1
PAO628	15	41 (pH 7.7)	ND	4.2	5.5
	60	86	1.4	8.4	<0.1
PAO630	15	<0.1	ND	1.6	12.5
	60	<0.1	0.7	15.3	1.1

[a] After growth under O_2 limitation. Growth medium was medium P + 26 μmol of L-arginine per ml.

[b] Expressed as micromoles of product formed per hour per milligram of protein. Parentheses indicate pH optimum. ND, Not determined.

[c] Determined on an amino acid analyzer.

alyzed by transduction. Bacteriophage G101 was grown on all Su^- strains (wild type and mutants) and on the Su^+ strain PAO529, a *hisII* derivative of PAO524. When selection was made for Arg^+ (Su^+), any $Su^- \times Su^-$ cross gave a much lower number of transductants than the $Su^+ \times Su^-$ crosses (see Table 6). The Su^- loci are thus closely linked. The recovery of Arg^+ recombinants was particularly low in Su^- (wild type) $\times$ Su^- (mutant) crosses, whereas some Su^- (mutant) $\times$ Su^- (mutant) crosses yielded Arg^+ recombinants at relatively high frequencies. Reciprocal crosses did not always give the same "prototroph reduction," e.g., in the case of PAO629 $\times$ PAO624, but there was no evidence of more than one transductional class of Su^- loci. In addition, the Su^- loci were linked to *hisII* in transduction (results not shown).

It seems likely that in all Su^- derivatives mutations have occurred in the structural gene for the catabolic ornithine carbamoyltransferase. Hence we consider the Su^- loci as *arcB* alleles.

DISCUSSION

The major problem in the isolation and genetic characterization of arginine dihydrolase mutants was the fact that these mutants were able to utilize arginine as the only carbon and nitrogen source and did not have a selectable phenotype. However, the efficient suppression of the *argF* function by a modified catabolic ornithine carbamoyltransferase gave a clear-cut phenotype (arginine prototrophy) which could be used in genetic experiments and allowed mapping of the suppressor locus *arcB1*(Su). The molecular properties of the suppressor enzyme suggest that *arcB1*(Su) lies in the structural gene for the catabolic ornithine carbamoyltransferase. Several suppressor-negative derivatives of the *arcB1*(Su) strain were obtained in independent experiments. All Su^- loci were closely linked to *arcB1*(Su), and no evidence for an unlinked "anti-suppressor" could be found.

Since NG was used to derive the suppressor strain, this mutant may carry more than one mutation within *arcB1*(Su) or in genes adjacent to *arcB*. Some transductions involving *arcB1*-(Su) gave strongly asymmetrical linkage values in reciprocal crosses (Tables 4 and 6). Multiple mutations in the *arcB* region might explain, in part, this phenomenon.

It has been known for some time that the arginine dihydrolase enzymes are induced when *Pseudomonas* cultures are grown in arginine media under limitation of oxygen (15, 23). In our experiments with *P. aeruginosa*, high activities of the catabolic ornithine carbamoyltransferase (Table 5) and arginine deiminase (data not shown) were obtained when cultures were grown with poor aeration in sealed flasks. The highest specific activities were found late in the stationary phase. Induction by oxygen limitation was more than 50-fold, whereas the addition of arginine increased enzyme levels by a factor of two. The dihydrolase enzyme molecules are not sensitive to oxygen in vitro, and there is no evidence for oxygen sensitivity in vivo. It cannot be excluded that the elevated enzyme levels observed late in the stationary phase are due to modification of existing enzyme molecules rather than de novo synthesis.

Several lines of evidence suggest that the arginine dihydrolase pathway is not the only arginine catabolic pathway in *P. aeruginosa*. (i) Mutant PAO630 lacks catabolic ornithine carbamoyltransferase activity in crude extracts as well as in permeabilized whole cells, but nevertheless grows on arginine at the wild-type growth rate provided oxygen is not limiting. (ii)

TABLE 6. *Transductional analysis of Su^- strains*[a]

Recipient and relevant genotype	Selection	G101 donor					
		PAO522	PAO622	PAO624	PAO627	PAO629	PAO529
PAO522 *arcB*$^+$(Su^-)	Arg^+	0	0	1	0	0	42
PAO622 *arcB2*(Su^-)	Arg^+	0	0	0	0	0	24
PAO624 *arcB4*(Su^-)	Arg^+	7	40	0	17	144	565
PAO627 *arcB7*(Su^-)	Arg^+	1	13	25	1	79	261
PAO629 *arcB9*(Su^-)	Arg^+	4	5	5	6	0	146
PAO529 *his-16 arcB1*(Su^+)	His^+	185	189	199	137	69	0

[a] The figures represent numbers of transductants per plate. Equal volumes of cell suspensions (approximately 2×10^9 cells per ml) and phage suspensions (10^{10} plaque-forming units per ml) were mixed. After adsorption for 30 min in TNM buffer (4), mixtures were concentrated twofold by centrifugation, and samples of 0.2 ml were plated. His^+ transductants were counted after 3 days of incubation at 37°C; Arg^+ transductants were counted after 5 days. All crosses were done in duplicate or in quadruplicate (when PAO529 was the donor or recipient). In a control experiment (data not given) it was shown that the *pur-136* marker in PAO622, PAO624, PAO627, and PAO629 could be transduced to Pur^+ at the normal frequencies with G101.PAO1.

The defect of ornithine carbamoyltransferase in PAO630 causes strong citrulline excretion during oxygen limitation (i.e., when arginine deiminase is induced), but not during aerobic growth, arginine being the growth substrate. By contrast, the wild-type PAO1 accumulates little citrulline under O_2 limitation. (iii) Recently, we have obtained a mutant blocked in arginine deiminase and catabolic ornithine carbamoyltransferase. This mutant still grows on arginine and does not excrete citrulline (A. Mercenier and D. Haas, unpublished data). (iiii) Mercenier et al. (12) have shown that *P. aeruginosa* is able to convert arginine to putrescine via agmatine and *N*-carbamoylputrescine. Putrescine can be degraded to succinate (24). We presume that this pathway is important in aerobic cultures growing on arginine.

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LITERATURE CITED

1. **Bartnik, E., and P. Weglenski.** 1974. Regulation of arginine catabolism in *Aspergillus nidulans*. Nature (London) **250**:590-592.
2. **Haas, D., and B. W. Holloway.** 1976. R Factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **144**:243-251.
3. **Haas, D., and B. W. Holloway.** 1978. Chromosome mobilization by the R plasmid R68.45: a tool in *Pseudomonas* genetics. Mol. Gen. Genet. **158**:229-237.
4. **Haas, D., B. W. Holloway, A. Schambock, and T. Leisinger.** 1977. The genetic organization of arginine biosynthesis in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **154**:7-22.
5. **Holloway, B. W.** 1969. Genetics of *Pseudomonas*. Bacteriol. Rev. **33**:419-433.
6. **Horn, F.** 1933. Über den Abbau des Arginins zu Citrullin durch Bacillus pyocyaneus. Hoppe-Seylers Z. Physiol. Chem. **216**:244-247.
7. **Legrain, C., and V. Stalon.** 1976. Ornithine carbamoyltransferase from *Escherichia coli* W. Purification, structure and steady-state kinetic analysis. Eur. J. Biochem. **63**:289-301.
8. **Legrain, C., V. Stalon, J. P. Noullez, A. Mercenier, J. P. Simon, K. Broman, and J. M. Wiame.** 1977. Structure and function of ornithine carbamoyltransferases. Eur. J. Biochem. **80**:401-409.
9. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
10. **Matsumoto, H., S. Ohta, R. Kobayashi, and Y. Terawaki.** 1978. Chromosomal location of genes participating in the degradation of purines in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **167**:165-176.
11. **Mee, B. J., and B. T. O. Lee.** 1967. An analysis of histidine requiring mutants in *Pseudomonas aeruginosa*. Genetics **55**:709-722.
12. **Mercenier, A., J. P. Simon, and V. Stalon.** 1978. Le catabolisme de l'arginine chez *Pseudomonas aeruginosa*. Arch. Intern. Physiol. Biochim. **86**:85-86.
13. **Mindich, L., J. Cohen, and M. Weisburd.** 1976. Isolation of nonsense suppressor mutants in *Pseudomonas*. J. Bacteriol. **126**:177-182.
14. **Ramos, F., V. Stalon, A. Piérard, and J. M. Wiame.** 1967. The specialization of the two ornithine carbamoyltransferases of *Pseudomonas*. Biochim. Biophys. Acta **139**:98-106.
15. **Sherris, J. C., J. G. Shoesmith, M. T. Parker, and D. Breckon.** 1959. Tests for the rapid breakdown of arginine by bacteria: their use in the identification of pseudomonads. J. Gen. Microbiol. **21**:389-396.
16. **Slade, H. D., C. C. Doughty, and W. C. Slamp.** 1954. The synthesis of high-energy phosphate in the citrulline ureidase reaction by soluble enzymes of *Pseudomonas* Arch. Biochem. Biophys. **48**:338-346.
17. **Stalon, V., C. Legrain, and J. M. Wiame.** 1977. Anabolic ornithine carbamoyltransferase of *Pseudomonas*. The bases of its functional specialization. Eur. J. Biochem. **74**:319-327.
18. **Stalon, V., F. Ramos, A. Piérard, and J. M. Wiame.** 1967. The occurrence of a catabolic and an anabolic ornithine carbamoyltransferase in *Pseudomonas*. Biochim. Biophys. Acta **139**:91-97.
19. **Stalon, V., F. Ramos, A. Piérard, and J. M. Wiame.** 1972. Regulation of the catabolic ornithine carbamoyltransferase of *Pseudomonas fluorescens*. A comparison with the anabolic transferase and with a mutationally modified catabolic transferase. Eur. J. Biochem. **29**:25-35.
20. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43**:159-271.
21. **Stanisich, V. A., and B. W. Holloway.** 1972. A mutant sex factor of *Pseudomonas aeruginosa*. Genet. Res. **19**: 91-108.
22. **Theil, E. C., G. W. Forsyth, and E. E. Jones.** 1969. Expression of the arginine regulon of *Escherichia coli* W: evidence for a second regulatory gene. J. Bacteriol. **99**:269-273.
23. **Thornley, M. J.** 1960. The differentiation of *Pseudomonas* from other gram-negative bacteria on the basis of arginine metabolism. J. Appl. Bacteriol. **23**:37-52.
24. **Voellmy, R., and T. Leisinger.** 1976. Role of 4-aminobutyrate aminotransferase in the arginine metabolism of *Pseudomonas aeruginosa*. J. Bacteriol. **128**:722-729.
25. **Voellmy, R., and T. Leisinger.** 1978. Regulation of enzyme synthesis in the arginine biosynthesis pathway of *Pseudomonas aeruginosa*. J. Gen. Microbiol. **109**: 25-35.
26. **Watson, J. M., and B. W. Holloway.** 1976. Suppressor mutations in *Pseudomonas aeruginosa*. J. Bacteriol. **125**:780-786.
27. **Watson, J. M., and B. W. Holloway.** 1978. Chromosome mapping in *Pseudomonas aeruginosa* strain PAT. J. Bacteriol. **133**:1113-1125.