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Pseudomonas aeruginosa Mutants Affected in Anaerobic Growth on Arginine: Evidence for a Four-Gene Cluster Encoding the Arginine Deiminase Pathway

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***Pseudomonas aeruginosa* PAO was able to grow in the absence of exogenous terminal electron acceptors, provided that the medium contained 30 to 40 mM L-arginine and 0.4% yeast extract. Under strictly anaerobic conditions (O_2 at <1 ppm), growth could be measured as an increase in protein and proceeded in a non-exponential way; arginine was largely converted to ornithine but not entirely consumed at the end of growth. In the GasPak anaerobic jar (Becton Dickinson and Co.), the wild-type strain PAO1 grew on arginine-yeast extract medium in 3 to 5 days; mutants could be isolated that were unable to grow under these conditions. All mutants (except one) were defective in at least one of the three enzymes of the arginine deiminase pathway (*arcA*, *arcB*, and *arcC* mutants) or in a novel function that might be involved in anaerobic arginine uptake (*arcD* mutants). The mutations *arcA* (arginine deiminase), *arcB* (catabolic ornithine carbamoyltransferase), *arcC* (carbamate kinase), and *arcD* were highly cotransducible and mapped in the 17-min chromosome region. Some mutations in the *arc* cluster led to low, noninducible levels of all three arginine deiminase pathway enzymes and thus may affect control elements required for induction of the postulated *arc* operon. Two fluorescent pseudomonads (*P. putida* and *P. fluorescens*) and *P. mendocina*, as well as one PAO mutant, possessed an inducible arginine deiminase pathway and yet were unable to grow fermentatively on arginine. The ability to use arginine-derived ATP for growth may provide *P. aeruginosa* with a selective advantage when oxygen and nitrate are scarce.**

The arginine deiminase pathway, one of the pathways involved in arginine catabolism in *Pseudomonas aeruginosa* (18, 30), generates 1 mol of ATP from the utilization of 1 mol of L-arginine (34). This pathway is induced under conditions of limited oxygen supply and, to a lesser extent, after depletion of the carbon and energy source (19).

Mutants that do not utilize arginine as the only carbon and nitrogen source under aerobic conditions can be isolated readily, but they are not defective in the structural genes of the arginine deiminase pathway (24; A. Mercenier, Ph.D. thesis, Université Libre de Bruxelles, Belgium, 1980; and our unpublished data). Mutants affected in this metabolic route have been obtained by indirect selection procedures. The second enzyme of the pathway, the catabolic ornithine carbamoyltransferase (EC 2.1.3.3), can be mutationally altered so that it suppresses a defect of the biosynthetic (anabolic) ornithine carbamoyltransferase, the *argF* gene product (8). Strains that have lost this suppressor activity lack the catabolic ornithine carbamoyltransferase (8). Such *arcB* mutants, when grown on arginine, excrete large amounts of citrulline during the stationary growth phase. A derivative of an *arcB* strain failing to excrete citrulline under these conditions has been found to be devoid of the first pathway enzyme, arginine deiminase (EC 3.5.3.6) (20). Both *arcA* and *arcB* mutants are able to grow aerobically with arginine as the sole C and N source (12).

The ATP generated by anaerobic metabolism of L-arginine can be used to promote motility (28) and to maintain the membrane potential of *P. aeruginosa* (3). Although the intracellular ATP concentration is relatively low when *P. aeruginosa* is incubated anaerobically with L-arginine as the only energy source (3), we show here that the organism is nevertheless capable of limited growth in a rich medium containing 40 mM L-arginine but no exogenous electron acceptors such as oxygen, nitrate or nitrite. Thus, the ATP formed from arginine via the deiminase pathway can be used for growth, and one commonly used definition of the genus *Pseudomonas*—an organism having a strictly respiratory type of metabolism (23)—is not entirely correct for *P. aeruginosa*.

Since the *arcA* and *arcB* mutants described previously (8, 20) could not grow anaerobically with arginine as the sole energy source, this provided us with a convenient method for the isolation of new mutants affected in the structural genes of the arginine deiminase pathway, including carbamate kinase (EC 2.7.2.2) and an additional function that might be required for anaerobic arginine transport.

(Preliminary results of this work have been communicated (35; A. Mercenier, C. Vander Wauven, V. Stalon, A. Piérard, and D. Haas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K109, p. 195.)

MATERIALS AND METHODS

Bacterial strains. All mutants used in this work were derived from *P. aeruginosa* PAO (see Table 1). Other wild-type bacterial species are also listed in Table 1.

Media and growth conditions. The *P. aeruginosa* strains were grown at 37°C, and the other *Pseudomonas* species

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TABLE 1. Bacterial strains

Strain	Genotype[a]	Method of construction or reference
P. aeruginosa		
PAO1	Wild type	15
PAO25(R68.45)	*argF10 leu-10*/Cb Tc Km Cma$^+$	9, 10
PAO505	*met-9011 amiE200*	36
PAO522	*pur-136 argF2*	11
PAO524	*pur-136 argF2 arcB1*(Su)	8
PAO534	*ilv-226 his-4 argA127 met-28 pro-82*	Haas collection
PAO630	*argF2 arcB9*	8
PAO977	*aru-180*	Defective in aerobic arginine uptake; Leisinger collection
PAO990	*argF2 arcB9 arcA1*	20
PAO6126	*ilv-226 his-4 argA127 pro-82*	Met$^+$ transductant of PAO534
Derivatives of PAO505		
PAO6102	*arcB6102*	Nitrosoguanidine-induced derivatives of PAO505 unable to grow anaerobically on arginine
PAO6107	*arcC6107*	
PAO6114	*arcA6114*	
PAO6116	*arcD6116*	
PAO6134	*arc-6134*	
PAO6138	*arcD6138*	
PAO6151	*arcB6151*	
PAO6161	*arcA6161*	
PAO6167	*arc-6167*	
PAO6170	*arcD6170*	
PAO6171	*arcC6171*	
PAO6186	*arc-6186*	
Other species		
P. mendocina		NCIB 10541
P. putida		ATCC 12633
P. putida		ATCC 25571
P. putida		IRC 204 (31)
P. fluorescens		ATCC 13525
M. vannielii		DSM 1224 (German Collection of Microorganisms, Göttingen, Germany)

[a] Genotype symbols: *amiE*, amidase; *arc*, inability to grow anaerobically on arginine as the energy source; *arcA*, arginine deiminase; *arcB*, catabolic ornithine carbamoyltransferase; *arcC*, carbamate kinase; *arcD*, anaerobic arginine uptake (?); *argF*, anabolic ornithine carbamoyltransferase; *aru*, aerobic arginine utilization; other symbols are the same as those for *Escherichia coli* (4). Plasmid phenotype: Cma$^+$, chromosome mobilizing ability (10).

were grown at 30°C. For aerobic growth, nutrient yeast broth, nutrient agar (33), and the minimal nitrogen salt-free medium 154 (31) were used; the latter contained C and N sources (e.g., L-arginine or L-glutamate) at concentrations of 20 mM and the required amino acid supplements at 100 μg/ml. The growth conditions of good aeration or limited oxygen supply (using 500-ml cultures in 1-liter hermetically sealed flasks) have been described previously (8, 18).

For anaerobic growth, either medium 154 or that of Ornston and Stanier (22) (OS medium) was used as a basal salts solution. The OS medium was prepared without nitriloacetate, and trace metal salts contained chloride rather than nitrate. Both 154 and OS media were strongly buffered (ca. 0.1 M phosphate buffer). This is important because 2 mol of NH_3 is liberated from arginine in the arginine deiminase pathway (34). The basal salts solution (154 or OS) was supplemented with 0.4% (wt/vol) Difco yeast extract and 30 or 40 mM L-arginine · HCl (YEA medium). For monitoring of anaerobiosis, 3 mM cysteine · HCl and 0.001% resazurin (boiled before use) were added. Since the yeast extract can provide the necessary carbon and nitrogen sources and cell constituents for *P. aeruginosa* and since arginine seems to serve only as an energy source during anaerobiosis, it was possible to replace the yeast extract by a semisynthetic mixture containing (final concentrations): 1% (wt/vol) Difco Casamino Acids, 3 mM L-cysteine · HCl; 1 mM L-tryptophane, 20 mM D-glucose (optional), 1 mM uracil, 1 mM adenine, 0.1 mM nicotinate, 0.1 mM pyridoxamine · HCl, 0.1 mM thiamine, 0.005% folate, and 0.001% resazurin. Cysteine and resazurin inhibited anaerobic growth of *P. aeruginosa* somewhat; therefore, they were omitted in quantitative growth experiments. The OS medium amended with 40 mM arginine plus 0.4% (wt/vol) yeast extract or the semisynthetic supplement described above contained ≤1 μM nitrate and ≤2 μM nitrite (L. Sigg, personal communication).

For routine genetic analysis, plates (YEA medium + 1.5% [wt/vol] agar) were poured under aerobic conditions and prewarmed to 37°C before use. Strains to be tested for anaerobic growth were streaked to single colonies. Plates were then incubated in an anaerobic jar (GasPak; Becton Dickinson & Co.). Since 1 to 2 h are required for an essentially anaerobic H_2-CO_2 atmosphere to become established in the GasPak system (27), there was sufficient time for induction of the arginine deiminase pathway enzymes during semi-anaerobic conditions (19). Best results were obtained with bacteria streaked from liquid cultures grown with limited aeration. A positive (strain PAO1) and a negative (strain PAO990) control were always included. Growth was scored after 3 to 6 days of incubation in the anaerobic jar.

Experiments designed to demonstrate growth of *P. aeruginosa* in the complete absence of respiration were done in an anaerobe chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.) containing N_2-H_2 (92:8) and O_2 at ≤4 ppm. YEA plates were poured in the anaerobe chamber and stored

for several days before use until the redox indicator resazurin was colorless. Liquid *P. aeruginosa* cultures containing induced levels of the arginine deiminase pathway enzymes were streaked to single colonies; the plates were incubated in a pressure cylinder filled with an N_2-CO_2-H_2 (90:5:5) mixture containing O_2 at <1 ppm after passage through an Oxisorb cartridge (Messer-Griessheim, Düsseldorf, West Germany). Incubation was at 37°C for 7 to 10 days.

For the preparation of anaerobic liquid YEA medium, the OS basal salts solution containing 0.4% yeast extract was freed of oxygen by boiling for 1 h and gassing with N_2-CO_2 (80:20). In the glove box, the buffer was distributed to 50-ml bottles fitted with airtight rubber caps. The gas phase was exchanged for N_2-CO_2-H_2 (90:5:5). The bottles were autoclaved, and the remaining YEA components (sterilized by filtration) were injected from concentrated oxygen-free solutions. The final medium volume was 10 ml. The inoculum (100 μl of a *P. aeruginosa* suspension grown with limiting oxygen supply) was introduced with a syringe. The bottles were incubated on a rotary shaker (180 rpm). As a biological control for strict anaerobiosis, the highly oxygen-sensitive methanogenic bacterium *Methanococcus vannielii* was cultivated in parallel; YEA medium was supplemented with 2% (wt/vol) sodium formate, 0.05% Na_2S, and 0.05% cysteine · HCl (17).

Growth measurements. Growth in 10-ml anaerobic cultures was stopped after 6 to 80 h by the addition of 2 ml of cold 3 M trichloroacetic acid. The precipitate was centrifuged at 10,000 × *g* at 4°C and washed twice with cold 0.5 M trichloroacetic acid. The pellet was solubilized in 1.5 ml of 0.66 M NaOH at 30°C for 1 day. Protein was then determined by a modified Lowry method (16). The protein content of microorganisms is 67 to 71% of their dry mass and quite independent of growth conditions (13).

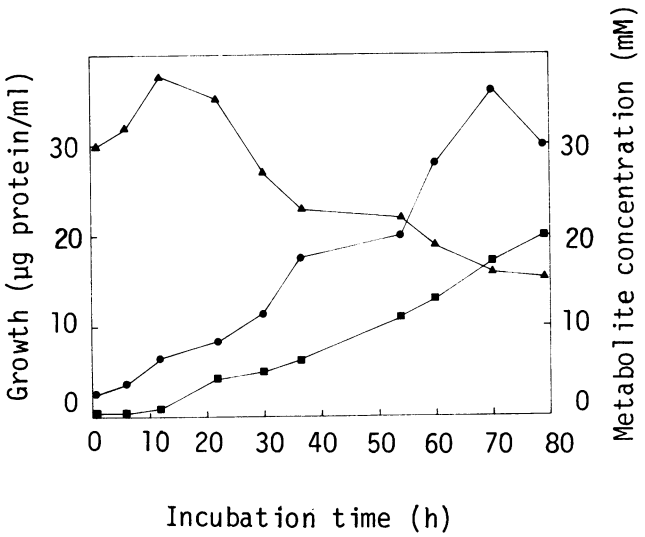

FIG. 1. Anaerobic growth of strain PAO1 with arginine as the energy source. OS medium containing 30 mM L-arginine and 0.4% yeast extract was distributed, in 10-ml portions, to 20 gas-tight bottles and inoculated with 100 μl of a PAO1 suspension as described in the text. The atmosphere contained O_2 at <1 ppm. At each sampling time, growth in two bottles was stopped by the addition of trichloroacetic acid; mean values are shown. Symbols: ●, protein concentration; ▲, arginine concentration in medium; ■, ornithine concentration in medium.

TABLE 2. Growth yield of PAO mutants in the absence of respiration[a]

Strain	Relevant genotype	Addition of 40 mM arginine	Growth yield[b] (μg of protein per ml)	Residual arginine[c] (mM)
PAO1	*arc*$^+$	−	7.2	1.5
		+	26	12
PAO505	*arc*$^+$	−	5.5	1.8
		+	21.5	10
PAO990	*arcAB*	−	7.1	2.0
		+	1.9	38
PAO6102	*arcB*	−	6.1	1.9
		+	2.0	39
PAO6107	*arcC*	−	3.9	2.7
		+	1.7	35
PAO6167	*arc-6167*	−	3.3	2.7
		+	2.2	40

[a] Incubation was in N_2-H_2-CO_2 (90:5:5) at 37°C for 3 days. OS medium with 0.4% yeast extract was used.
[b] The inoculum gave 2.8 μg of protein per ml.
[c] Assayed in culture supernatant.

Assay of amino acids in culture supernatants. Arginine was assayed colorimetrically by the method of Micklus and Stein (21). Ornithine was converted enzymatically to citrulline, which was measured by the colorimetric method previously described (32). The assay mixture contained 50 mM EDTA (sodium salt) (pH 8.5), 10 mM carbamoyl phosphate (lithium salt; Sigma Chemical Co.), 0.1 to 1.0 μmol of L-ornithine, and 20 U of purified *Streptococcus faecalis* ornithine carbamoyltransferase (kindly provided by V. Stalon) in a final volume of 2.0 ml. The incubation was at 37°C for 30 min.

Enzyme assays. Activities of arginine deiminase, catabolic ornithine carbamoyltransferase, carbamate kinase, and adenylate kinase (EC 2.7.4.3) were determined as previously described (19). The assays for acetate kinase (EC 2.7.2.1) and carbamoylphosphate synthetase (formerly EC 2.7.2.9, now EC 6.3.5.5) have been described previously (2, 26). Specific enzyme activities are expressed as micromoles of product formed per hour per milligram of protein (19).

Mutant isolation. After mutagenesis of strain PAO505 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (36), cells were plated on nutrient agar and incubated aerobically. Approximately 10,000 colonies were screened by replica plating onto YEA medium and anaerobic incubation in the GasPak system; 81 mutants were unable to grow under these conditions. They were characterized by assays for the arginine deiminase pathway enzymes. Twelve *arc* mutants were kept for further study (Table 1).

Genetic techniques. Plate matings, the construction of R68.45 donor strains, and G101 transductions have been previously described (9–11, 33). Linkage of newly isolated *arc* mutations to the *argF* suppressor locus *arcB1*(Su) (8) was tested as follows. In the *arc* mutant (e.g., PAO6102), the *met-9011* marker was replaced by the linked *argF2* mutation, using PAO522(R68.45) as a donor (8). In G101 transductions with the *arcB1*(Su) donor PAO524, selection was made for *argF*$^+$ recombinants (arginine prototrophy); the presence of an *arc* mutation in the recombinants was tested on YEA plates incubated anaerobically.

RESULTS

Anaerobic growth of *P. aeruginosa* on arginine. The wild-type PAO1 was able to grow anaerobically on plates containing 30 to 40 mM L-arginine and an enriching supplement; 0.4% yeast extract was best (YEA medium). The organism

TABLE 3. Enzymatic and genetic analysis of PAO mutants impaired in anaerobic growth on arginine

Strain	Relevant genotype	Enzyme[a] sp act (μmol/h per mg of protein)			Conjugational linkage[b] (%) of *arc* to:		Cotransduction[c] (%) of *arc* with *arcB1*(Su)
		ADI	cOTC	CK	*hisII*	*argA*	
PAO1	*arc*$^+$	24	502	232			
Group I							
PAO6114	*arcA*	3	633	257			97
PAO6161	*arcA*	1	488	182			95
PAO6102	*arcB*	26	16	140	98	96	98
PAO6151	*arcB*	23	9	127			99
PAO6171	*arcC*	8	338	1	90	96	93
PAO6107	*arcC*	29	522	4			91
PAO6167	*arc-6167*	1	3	18	94	93	98
PAO6186	*arc-6186*	2	23	22			99
Group II							
PAO6116	*arcD*	42	852	291			93
PAO6138	*arcD*	20	352	161	92	96	87
PAO6170	*arcD*	23	370	172			96
Group III							
PAO6134	*arc-6134*	39	377	523	<1	<1	<1

[a] ADI, Arginine deiminase; cOTC, catabolic ornithine carbamoyltransferase; CK, carbamate kinase.
[b] The *arc* mutants were made R68.45$^+$ and crossed with PAO6126 as the recipient; selection was made for *hisII*$^+$ or *argA*$^+$ and the presence of *arc* was scored (no anaerobic growth on YEA plates). A total of 250 to 300 recombinants were tested.
[c] The *arc* mutants were rendered *argF* (see the text) and transduced with a G101 lysate prepared on the *arcB1*(Su) strain PAO524. Selection was made for *arcB1*(Su) (arginine prototrophy); the presence of *arc* was scored.

was incubated in an atmosphere of N_2-H_2-CO_2 generated by the GasPak system (27). Arginine was essential for growth; yeast extract alone supported only very faint growth. Incubation at 37°C for 3 to 5 days was required for single colonies to appear. The previously described *arcA* and *arcB* mutants blocked in the arginine deiminase pathway (8, 20) could not grow anaerobically with arginine as the energy source but could do so when nitrate (100 mM) was supplied. Thus, it is unlikely that the traces of nitrate or nitrite found in the YEA medium (see above) could account for the anaerobic growth of *P. aeruginosa*.

Yeast extract could be replaced by a semisynthetic mixture of Casamino Acids, a purine, a pyrimidine, glucose (not essential but slightly stimulatory), and vitamins (see above), but growth was poorer than in YEA medium. Attempts to simplify the medium, e.g., by using a synthetic mixture of amino acids, failed; growth was too weak to be of practical value.

The GasPak atmosphere contains traces (<0.4%) of oxygen (27). For a demonstration that growth was still possible in the absence of these traces of oxygen, strain PAO1 was incubated in YEA medium in an atmosphere of N_2-CO_2-H_2 (90:5:5) containing O_2 at <1 ppm that supported growth of the oxygen-sensitive methanogen *M. vannielii*. Growth of strain PAO1 was very slow and not exponential (Fig. 1). Cells formed clumps and filaments; hence, a protein assay had to be used to measure growth. At the onset of growth (12 h), the arginine concentration in the medium increased (Fig. 1). We ascribe this reproducible increase to the action of exoproteases, which are derepressed when energy sources are limiting (37) and which may liberate arginine from peptides present in yeast extract. As growth proceeded, arginine was utilized and ornithine accumulated in the medium (Fig. 1). At the end of growth (70 h), arginine was not consumed entirely, indicating that some other component in the medium had become limiting (Fig. 1 and Table 2). The final pH was 6.9; thus, the NH_3 released from arginine was not a problem. After anaerobic growth, the cell mass as measured by the protein assay had increased ca. 10-fold from a 1% inoculum (Fig. 1 and Table 2). Growth was largely dependent on added arginine (Table 2) and on the presence of H_2 in the atmosphere (data not shown). On solid YEA medium, single colonies remained microscopic in size under the same stringent oxygen-free conditions.

A functional arginine deiminase pathway was necessary but evidently not sufficient for fermentative growth of *Pseudomonas* spp. *P. mendocina* (NCIB 10541), *P. fluorescens* (ATCC 13525), and three strains of *P. putida* (ATCC 12633, ATCC 25571, and IRC 204), which all have an inducible arginine deiminase pathway (30), were unable to grow on YEA plates in the GasPak system but showed lush growth in the presence of air.

Isolation and characterization of mutants unable to grow anaerobically on arginine. Guided by the growth behavior of existing *arcAB* mutants, we isolated new nitrosoguanidine-induced mutants impaired in arginine fermentation. Eighty-one clones were obtained that could not grow on YEA plates in the GasPak system. Most of these lacked one enzyme of the arginine deiminase pathway: arginine deiminase (*arcA*), catabolic ornithine carbamoyltransferase (*arcB*), or carbamate kinase (*arcC*). Some strains had lost two enzyme activities (the deiminase and the kinase), and a few mutants had strongly reduced, noninducible activities of all three enzymes. These various mutants constitute group I; representative strains are listed in Table 3. Growth tests in liquid media confirmed that these *arc* mutants were unable to degrade arginine anaerobically and to utilize it for growth (Table 2). All *arc* mutations of group I were 91 to 99% cotransducible with the *argF* suppressor locus *arcB1*(Su), which has been mapped previously at 17 min on the PAO chromosome map, between *hisII* and *argA* (8; Table 3). The *arcB1*(Su) locus specifies a modified catabolic ornithine carbamoyltransferase working in both the anabolic and the catabolic directions. Selection for this suppressor (i.e., arginine prototrophy) is possible in crosses when the donor and the recipient carry the same *argF* allele (8). High conjuga-

tional linkage of *arcB*, *arcC*, and *arc-6167* to *hisII* and *argA* (Table 3) affords further proof for the location of an *arc* cluster in the 17-min chromosome region.

Twenty-three mutants were unable to grow anaerobically on arginine but nevertheless could induce all three enzymes of the arginine deiminase pathway to wild-type levels. Because the corresponding *arc* mutations in three representative strains were highly cotransducible with *arcB1*(Su) and thus close to the *arcABC* cluster, they were placed into a second group, tentatively called *arcD* (Table 3). Arginine consumption by *arcD* mutants was measured in oxygen-limited and well-aerated cultures (Table 4). Whereas the wild-type PAO1 degraded 20 mM L-arginine totally during oxygen-limited growth, less than 20% of the arginine was utilized by *arcD* mutants under the same conditions (Table 4). During good aeration, the *arcD* mutants appeared to degrade arginine normally (Table 4) and could utilize arginine as the only C and N source. This is possible because *arc* mutants possess several additional catabolic pathways that permit aerobic arginine utilization (12, 30). Conversely, a mutant defective in aerobic arginine uptake and utilization, PAO977 (*aru-180*), was able to degrade arginine entirely during oxygen-limited growth (Table 4) and to grow anaerobically on YEA plates. From this we conclude that *arcD* is not involved in aerobic arginine transport.

One mutant, PAO6134, possessed the arginine deiminase pathway enzymes (Table 3), consumed arginine during oxygen-limited growth (Table 4), and yet was not capable of fermentative growth on arginine. The *arc-6134* mutation was unlinked to the *arcABCD* cluster (Table 3). Strain PAO6134 was the only representative of group III (Table 3); it behaved like *P. putida*, *P. fluorescens*, and *P. mendocina* with respect to the absence of fermentative growth on arginine.

Further characterization of carbamate kinase mutants. In the *arcC* mutants, only carbamate kinase was impaired, whereas carbamoylphosphate synthetase, adenylate kinase, and acetate kinase were present at wild-type levels (data not shown). This result is consistent with the previous enzymatic characterization of carbamate kinase (1).

Like the wild-type strain, the *arcC* mutants excreted vast amounts of ornithine when grown in the presence of arginine in poorly aerated cultures; after consumption of 20 mM L-arginine, 81% of it was recovered as ornithine in the growth medium.

TABLE 4. Arginine consumption by *arc* mutants

Strain	Relevant genotype	% Residual arginine after 24 h of:	
		Oxygen-limited growth[a]	Aerobic growth[b]
PAO1	Wild type	<1	4
PAO977	*aru-180*	<1	90
PAO6116	*arcD*	92	2
PAO6138	*arcD*	90	18
PAO6170	*arcD*	83	1
PAO6134	*arc-6134*	<1	<1

[a] When cultures grown in 0.4% yeast extract with limiting aeration had reached the stationary phase, 20 mM L-arginine was added and incubation was continued for 24 h. This procedure prevents arginine consumption by cells during the initial aerobic growth phase.

[b] Cultures were grown in 0.4% yeast extract plus 20 mM L-arginine with vigorous shaking.

DISCUSSION

The utilization of arginine as an energy source for growth is well documented in a variety of microorganisms, e.g., *Mycoplasma* spp. (7), *Bacillus licheniformis* (6), *Halobacterium halobium* (14), and *S. faecalis* (29). In all of these organisms, the arginine deiminase pathway (sometimes also called the arginine dihydrolase pathway) is responsible for the formation of ATP from arginine. It has been known for a long time that *P. aeruginosa* can derive energy from arginine for motility (28). Yet there was no evidence that this energy can also be used for growth of *P. aeruginosa*, an organism classified as a strict aerobe (23).

Two observations prompted us to look for fermentative growth of *P. aeruginosa*. (i) The arginine deiminase pathway is strongly induced by different conditions of energy depletion such as shortage of terminal electron acceptors (19). This suggested that under these conditions, substrate level phosphorylation might produce sufficient ATP for growth, provided that high arginine concentrations were available in the medium. (ii) *P. aeruginosa* is able to colonize the intestinal tract, an essentially anaerobic, nutrient-rich environment. Among healthy individuals, 4 to 12% are *P. aeruginosa* carriers; this percentage is higher in stools from surgical patients receiving antibiotic treatment (25).

Our experiments have demonstrated that arginine fermentation can be utilized for limited growth of strain PAO. Two different systems were used to assess anaerobic growth with arginine as the only energy source. First, the GasPak anaerobic jar, which is widely used for the cultivation of anaerobic microorganisms of medical interest (27) but does not allow growth of some oxygen-sensitive methanogens, provided a convenient system for the isolation of *arc* mutants of strain PAO. The wild-type strain PAO1 grew to single colonies on YEA plates in ca. 5 days, whereas *arc* mutants could not grow. These mutants were not killed during incubation in the GasPak jar because they started growing rapidly as soon as air was supplied. The second anaerobic system, an anaerobic glove-box for manipulation and a pressure cylinder for incubation of cultures, is routinely used for the propagation of methanogens in our laboratory (17). With such stringent oxygen-free conditions, we could demonstrate a ca. 10-fold increase in protein (i.e., biomass) when strain PAO1 was incubated in liquid YEA medium. It was not feasible to correlate this increase in biomass with cell numbers because cells formed filaments and clumps. The shape of the growth curve (Fig. 1) and the fact that only microcolonies appeared on plates may mean that growth of *P. aeruginosa* was unbalanced when oxygen was completely eliminated. Because none of the *arc* mutants tested in this anaerobic system gave any growth, i.e., increase in protein (Table 2), it is clear that the arginine deiminase pathway plays a key role in the utilization of arginine as an energy source. The isolation of carbamate kinase mutants confirms that the synthesis of ATP from ADP and carbamoylphosphate is an essential step in the deiminase pathway and is required for fermentative growth in the GasPak system. In *arcC* mutants—as in the wild type—most of the arginine was converted to ornithine during oxygen-limited growth (Fig. 1); hence, the *arc* pathway contributes little towards the utilization of arginine as a C source. Furthermore, mutants that cannot utilize ornithine as a C and N source (24, 36) were able to grow by virtue of arginine fermentation (data not shown). Those pathways that are involved in the aerobic utilization of arginine as a C and N source (12, 30) apparently do not operate during anaerobiosis since *arc* mutants could not break down arginine anaerobically (Table 2).

For fermentative growth of *P. aeruginosa*, a rich medium was essential; 0.4% yeast extract was optimal. We think that the main function of yeast extract is to provide most, if not all, necessary cell constituents. It is not known which factor(s) in the YEA medium became limiting in the station-

ary phase (Fig. 1), but the limiting factor was not arginine.

Yeast extract contains amino acids, and in theory these could be used for energy production via "Stickland reactions" (5); however, we obtained no evidence for pairs of amino acids being utilized by *P. aeruginosa*. First, amino acid mixtures (either Casamino Acids or purely synthetic mixtures) were ineffective replacements for yeast extract. Second, all mutants unable to grow anaerobically in YEA medium were affected in the *arcABC* genes or in the closely linked *arcD* locus, which may be needed for anaerobic arginine uptake (see below). The one exception found, strain PAO6134, indicates that the presence of the arginine deiminase pathway alone is not sufficient for fermentative growth and that additional unknown functions must be present.

The *arcD* mutants (group II in Table 3) represent a novel type of defect. They were impaired in anaerobic arginine catabolism although they had induced levels of the arginine deiminase pathway enzymes; they could utilize arginine normally as a C and N source for aerobic growth. It appears that *arcD* mutants could not transport arginine into the cell during oxygen limitation. Alternatively, they might lack some cofactor needed for the function of the deiminase pathway in vivo. The *arcD* locus was strongly linked with *arcABC* and remote from the *aru-180* mutation which lies in the 90-min chromosome region (D. Haas, unpublished data) and affects aerobic arginine uptake (T. Leisinger, unpublished data). Therefore, *arcD* does not seem to specify a major arginine uptake system. However, there might be a special arginine transport system operating during oxygen limitation, and this system might be blocked in the *arcD* mutants.

In transductional and conjugational crosses, selection for Arc^+ (i.e., anaerobic growth on YEA plates) was not possible. Genetic mapping therefore relied mostly on the use of the selectable *argF* suppressor *arcB1*(Su). With the exception of *arc-6134*, all *arc* loci were tightly clustered. Recent cloning experiments have confirmed the existence of an *arcABCD* cluster (M. Rella, A. Mercenier, and D. Haas, unpublished data). The fact that in some mutants (PAO6167, PAO6186) all three enzymes were present at very low, noninduced levels suggests the existence of control elements linked to the putative *arcABCD* operon and required for induction.

P. aeruginosa was the only fluorescent pseudomonad found that could grow fermentatively with arginine. Under very stringent anaerobic conditions, only a small number of mass doublings took place (Fig. 1), and we did not attempt to obtain prolonged growth by serial transfers into fresh anaerobic YEA medium. We feel that *P. aeruginosa* would not be a competitive microorganism in such an extreme environment. However, as shown by our tests in the GasPak system, fermentative growth on arginine may give *P. aeruginosa* a considerable selective advantage in moderately anaerobic environments and, in certain cases, may contribute to the pathogenicity of the bacterium.

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