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Regulation of Enzyme Synthesis in the Arginine Deiminase Pathway of *Pseudomonas aeruginosa*

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The three enzymes of the arginine deiminase pathway in *Pseudomonas aeruginosa* strain PAO were induced strongly (50- to 100-fold) by a shift from aerobic growth conditions to very low oxygen tension. Arginine in the culture medium was not essential for induction, but increased the maximum enzyme levels twofold. The induction of the three enzymes arginine deiminase (EC 3.5.3.6), catabolic ornithine carbamoyltransferase (EC 2.1.3.3), and carbamate kinase (EC 2.7.2.3) appeared to be coordinate. Catabolic ornithine carbamoyltransferase was studied in most detail. Nitrate and nitrite, which can replace oxygen as terminal electron acceptors in *P. aeruginosa*, partially prevented enzyme induction by low oxygen tension in the wild-type strain, but not in *nar* (nitrate reductase-negative) mutants. Glucose was found to exert catabolite repression of the deiminase pathway. Generally, conditions of stress, such as depletion of the carbon and energy source or the phosphate source, resulted in induced synthesis of catabolic ornithine carbamoyltransferase. The induction of the deiminase pathway is thought to mobilize intra- and extracellular reserves of arginine, which is used as a source of adenosine 5'-triphosphate in the absence of respiration.

Two pathways participate in arginine catabolism in *Pseudomonas aeruginosa*. In the arginine deiminase pathway, arginine is degraded via citrulline to ornithine and carbamoylphosphate, which serves to generate ATP from ADP (13, 14). This pathway was found to provide energy for motility in a fluorescent *Pseudomonas* sp. strain under anaerobic conditions (11). A second catabolic pathway has been characterized recently; it involves the decarboxylation of arginine to agmatine, which is converted to *N*-carbamoylputrescine and putrescine (8, 9).

A mutant lacking the catabolic ornithine carbamoyltransferase, the second enzyme of the arginine deiminase pathway, is able to grow aerobically on arginine as the only carbon and nitrogen source at the same rate as the wild-type strain (4). Thus, we have suggested (4) that the arginine deiminase pathway is not primarily concerned with the utilization of arginine.

In this paper, we describe the regulation of this pathway, as determined by studying enzyme formation under a variety of growth conditions in the presence of oxygen and other terminal electron acceptors. It appears that depletion of terminal electron acceptors leads to an induction of the deiminase pathway, enabling the cells to generate energy from arginine.

MATERIALS AND METHODS

Bacterial strains and media. All mutants were derived from *P. aeruginosa* strain PAO1 (5). These strains were routinely grown at 37°C on minimal nitrogen salt-free medium 154 (13) supplemented with trace elements as follows: 28 mg of H_3BO_3 per liter, 7.5 mg of $Na_2MoO_4 \cdot 2H_2O$ per liter, 2.4 mg of $ZnSO_4$ per liter, and 2.5 mg of $CuSO_4 \cdot 5H_2O$ per liter. Carbon and nitrogen sources were added at concentrations of 25 mM each after sterilization. NO_3^- and NO_2^- concentrations were 100 and 10 mM, respectively, as suggested by Williams et al. (19). For limitation of growth by phosphate, the phosphate in the medium was replaced by 50 mM Tris-hydrochloride buffer (pH 7.0).

Growth of bacteria. Cells were grown in 1.3 liters of medium in a 2-liter microfermentor (Biolafitte, Maisons-Lafitte, France). Oxygen tension was measured with a sterilizable oxygen electrode, which was calibrated in sterile medium before inoculation. The system was allowed to equilibrate with air under agitation (200 rpm), and this oxygen tension was designated as 100%. Aerobic conditions were achieved by stirring the culture at a constant rate of 200 rpm, and air was sparged through the culture vessel to maintain air saturation (i.e., 100% oxygen tension). Exponentially growing cultures were used as inocula; the initial cell density in the fermentor was approximately 3×10^7 cells per ml.

Growth limitation experiments. In experiments with a limiting essential nutrient, there was a linear

relationship between the bacterial cell concentration and the particular nutrient in the culture. For carbon-limited growth 8 mM pyruvate was used; 0.2 mM phosphate was used for phosphate-limited growth. The conditions were chosen to give 3×10^8 to 4×10^8 cells per ml in starved cultures.

Preparation of cell extracts. Samples of the culture were pumped out of the fermentor with a peristaltic pump and chilled on ice, and 200 μg of chloramphenicol per ml was added. After the bacteria were harvested by centrifugation at 4°C, they were washed once with 0.9% (wt/vol) NaCl. Cell extracts were prepared as described previously (4).

Assay of catabolic ornithine carbamoyltransferase activity in cell extracts. Catabolic ornithine carbamoyltransferase was assayed by colorimetric determination of the citrulline formed from ornithine and carbamoylphosphate, as previously described (4).

Assay of arginine deiminase. Arginine deiminase enzyme was also assayed by colorimetric determination of citrulline. The standard incubation mixture contained the following (in 2 ml): 200 μmol of citrate-NaOH buffer (pH 5.5), 10 μmol of $MnCl_2$, 10 μmol of arginine, and extract. The reaction was started with arginine and stopped after 15 min at 37°C by adding 2.0 ml of 1 N hydrochloric acid.

Carbamate kinase determination. Carbamate kinase was assayed in the direction of ATP synthesis. The assay mixture (1 ml) contained 50 mM citrate-NaOH (pH 6.15), 40 mM $MgCl_2$, 7.5 mM ADP, 5 mM carbamoylphosphate, and cell extract. The reaction was started by adding ADP and stopped after 15 min of incubation by heating at 100°C for 3 min. The interfering adenylate kinase activity was determined under these same conditions in the absence of carbamoylphosphate. ATP formation was measured in a coupled assay mixture containing glucose and hexokinase, which resulted in the formation of glucose 6-phosphate; this compound was detected by the conversion of NADP to NADPH by glucose 6-phosphate dehydrogenase. The net increase in absorbance at 340 nm was measured with a Beckman DU spectrophotometer. The reaction mixture (1.0 ml) for the coupled assay contained 75 mM Tris-hydrochloride (pH 7.5), 0.37 mM NAD, 8 mM $MgCl_2$, 300 mM glucose, 6 U of yeast hexokinase (Boehringer), and 30 U of yeast glucose-6-phosphate dehydrogenase (grade I; Boehringer). The reaction was initiated by adding the sample containing ATP, and incubation was at 30°C for 60 min. The assay was proportional to ATP concentration from 0.01 to 0.15 μmol. The amount of carbamate kinase activity was obtained after subtraction of the interfering adenylate kinase activity, which appeared constitutive under the growth conditions examined (specific activity, approximately 10 μmol/h per mg of protein).

Protein concentrations. The protein concentrations of extracts were estimated by the method of Lowry et al. (7).

Specific activities. Specific activities are expressed as the amount of enzyme that catalyzed the formation of 1 μmol of product per h per mg of protein.

Arginine consumption. In some experiments, the amount of arginine consumed during the growth process was determined by using an amino acid analyzer, as previously described (8).

RESULTS

Effect of oxygen tension on enzyme formation in the arginine deiminase pathway. The levels of the arginine deiminase pathway enzymes in *P. aeruginosa* cultures grown aerobically were difficult to reproduce. The specific activities of arginine deiminase and catabolic ornithine carbamoyltransferase appeared to depend on the form of the flask, the volume of the culture medium, and the culture density at harvesting time. The rate of catabolic ornithine carbamoyltransferase synthesis was low during exponential growth and at the end of the exponential phase in a variety of growth media with or without arginine (Table 1). In a minimal medium containing arginine and glutamate, decreasing the oxygen tension in the culture medium led to a strong induction of the arginine deiminase pathway (Fig. 1). When the air supply was cut off, the oxygen tension in the medium fell below the limit of detection (i.e., <1% saturation) within a few minutes, although the culture remained in contact with air at atmospheric

TABLE 1. *Regulation of specific catabolic ornithine carbamoyltransferase activities in strain PAO1*

Growth medium[a]	Oxygen tension[b]	Sp act (μmol of citrulline formed per h per mg of protein)
Glutamate	High	22[c]
Arginine	High	14
Glutamate + arginine	High	15
Glutamate	Low	740
Citrate + NH_4^+	Low	448
Glucose + NH_4^+	Low	270
Glutamate + arginine	Low	1,212
Pyruvate + arginine	Low	850
Succinate + arginine	Low	770
Citrate + arginine	Low	560
Fumarate + arginine	Low	486
Glucose + arginine	Low	320

[a] Medium 154 was supplemented with a carbon source (25 mM) and/or a nitrogen source (25 mM).

[b] High oxygen tension means air saturation; cultures were harvested during the exponential growth phase. Low oxygen tension means <1% saturation. Cultures were grown aerobically to a concentration of approximately 2.5×10^8 cells per ml and then incubated without aeration for 2.5 h, as described in the legend to Fig. 1.

[c] Basal enzyme level. Some of the enzyme activity may have been due to anabolic ornithine carbamoyltransferase, which is formed at a high rate in medium containing glutamate (17).

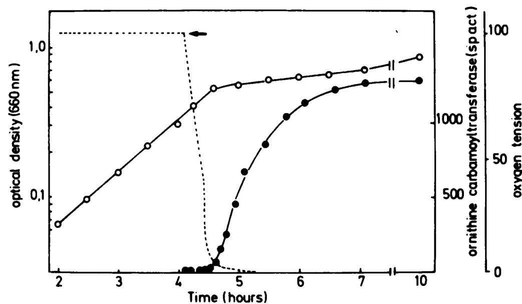

FIG. 1. *Induction of catabolic ornithine carbamoyltransferase during growth of P. aeruginosa strain PAO1 on medium containing 25 mM glutamate and 25 mM arginine under low oxygen tension. The dashed line shows the oxygen tension. Symbols: O, growth given as the extinction at 660 nm; ●, catabolic ornithine carbamoyltransferase specific activity. Aeration was stopped at the time indicated by the arrow.*

pressure. As soon as the oxygen tension became undetectable, all three enzymes of the pathway (arginine deiminase [EC 3.5.3.6], catabolic ornithine carbamoyltransferase [EC 2.1.3.3], and carbamate kinase [EC 2.7.3.2]) were formed rapidly, and coordinate synthesis of these enzymes was observed (Fig. 2). In the experiments described below, the levels of catabolic ornithine carbamoyltransferase and arginine deiminase were assayed under a variety of conditions, but carbamate kinase was not studied in such detail.

When chloramphenicol (final concentration, 200 μg/ml) was added to a culture 30 min after the interruption of the oxygen supply, the levels of arginine deiminase and catabolic ornithine carbamoyltransferase did not increase (data not shown). Thus, de novo protein synthesis is required for the induction of the deiminase pathway. In a culture induced for the arginine deiminase pathway by low oxygen tension, restoration of aeration resulted in a resumption of fast growth, and catabolic ornithine carbamoyltransferase formation was repressed (data not shown).

Effect of arginine. Induction of catabolic ornithine carbamoyltransferase occurred under low oxygen tension even in the absence of arginine (Table 1). However, the addition of 10 mM arginine to an induced culture of strain PAO1 (growing on medium containing citrate and NH_4^+) increased the level of this enzyme by a factor of two (Fig. 3). Maximum enzyme formation was observed during growth on medium containing glutamate and arginine about 2 h after the air supply was cut off (Table 1). Slow growth continued for 6 h under low oxygen tension. When growth had stopped, 40% of the initial glutamate and 40% of the initial arginine were still present in the medium, and the consumption of these two compounds continued for about 10 h without change in the bacterial mass. Although *P. aeruginosa* grows well on arginine as the only nutrient (17), cell lysis often occurred under high oxygen tension, and hence experiments in which arginine was used as the sole carbon and nitrogen source were abandoned. Catabolite repression of catabolic ornithine carbamoyltransferase occurred when *P. aeruginosa* was cultivated in media containing arginine and other carbon sources (Table 1). The lowest level of catabolic ornithine carbamoyltransferase was obtained in the presence of glucose; fumarate and citrate gave specific activities which were about 40 to 50% of the maximum level obtained with medium containing glutamate plus arginine.

Effect of nitrate and nitrite. *P. aeruginosa* is able to grow anaerobically with nitrate or nitrite as terminal electron acceptor (15, 16). This fact was exploited to study the regulation of the deiminase pathway during different modes of respiration. In the presence of nitrate, a shift from full aerobiosis to low oxygen tension

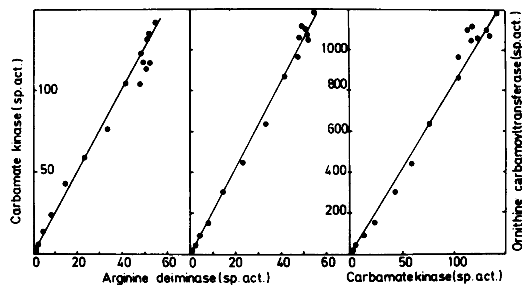

FIG. 2. *Coordinate regulation of the enzymes of the arginine deiminase pathway in an experiment identical to that described in the legend to Fig. 1.*

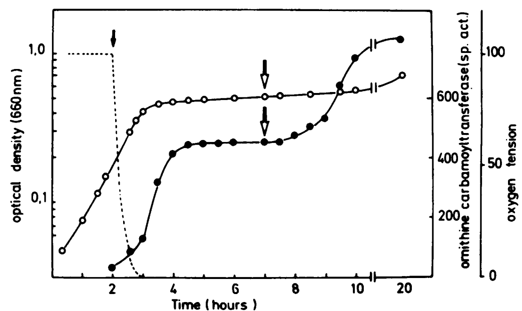

FIG. 3. *Induction of catabolic ornithine carbamoyltransferase during growth of P. aeruginosa on medium containing 25 mM citrate and 20 mM NH_4^+ under low oxygen tension. At the times indicated by the open arrows, 10 mM arginine was added as a supplement to the growth medium. Symbols: O, growth given as the extinction at 660 nm; ●, catabolic ornithine carbamoyltransferase specific activity.*

caused only a slight reduction in the doubling time; the catabolic ornithine carbamoyltransferase level increased for 1 h after the oxygen tension became zero (Fig. 4). However, the maximum level of activity with nitrate was much lower than the maximum level obtained without nitrate, and after 1 h the specific activity decreased slowly to a final level corresponding to about 20% of the maximally induced activity (Fig. 1 and 4).

With nitrite as the electron acceptor, some inhibition of catabolic ornithine carbamoyltransferase induction was also observed (Table 2). We may conclude that both nitrate and nitrite prevent induction of the arginine deiminase pathway, but do so less efficiently than oxygen.

van Hartingsveldt et al. (15, 16) isolated mutants of strain PAO impaired in the dissimilatory nitrate reductase (*nar*) and anaerobic growth (*ana*). In four *nar* mutants tested, nitrate did not prevent the strong induction of catabolic ornithine carbamoyltransferase under low oxygen tension (Table 2). Similarly, in an *anaA* mutant, which has nitrate and nitrite reductase activities but does not grow anaerobically (16), nitrate and nitrite failed to interfere with catabolic ornithine carbamoyltransferase induction. These findings indicate that the terminal electron acceptors themselves are not directly involved in the regulation of the deiminase pathway.

Effect of nutrient depletion. If induction of catabolic ornithine carbamoyltransferase occurs in *P. aeruginosa* during removal of oxygen as a result of energy limitation, then other conditions which produce an energy deficiency might also be expected to lead to induction of the arginine deiminase pathway. *P. aeruginosa* was grown aerobically with limiting amounts of either pyruvate (8 mM) as the only carbon source or inorganic phosphate (0.2 mM); growth came to a stop after about five generations. The level of ornithine carbamoyltransferase, which was low during exponential growth, increased during the first 30 min of the stationary phase and then remained at this level for at least 4 h (Table 3).

DISCUSSION

What is the physiological role of the arginine deiminase pathway in *P. aeruginosa*? Previous work (4, 8) has shown that under aerobic conditions a block in catabolic ornithine carbamoyltransferase does not prevent the utilization of arginine as the only nutrient; in fact, arginine can be degraded to putrescine via the arginine decarboxylase pathway. Since ATP is one of the end products of the deiminase pathway, this pathway can provide energy in microorganisms such as *Bacillus licheniformis* (3), *Streptococcus faecalis*, and *Mycoplasma arthritidis* (1).

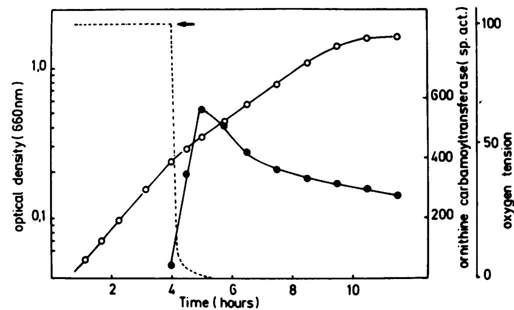

FIG. 4. *Induction of catabolic ornithine carbamoyltransferase during growth of P. aeruginosa on medium containing 25 mM glutamate, 25 mM arginine, and 100 mM nitrate under low oxygen tension. Aeration was stopped at the time indicated by the arrow. Symbols: ○, growth given as extinction at 660 nm; ●, catabolic ornithine carbamoyltranserase specific activity.*

TABLE 2. *Regulation of specific catabolic ornithine carbamoyltransferase activities in cultures grown on medium containing glutamate and arginine under low oxygen tension*

Strain[a]	Relevant genotype	Electron acceptor	Sp act (μmol of citrulline per h per mg of protein)[b]
PAO1	Wild type	NO_3^-	333
PAO1	Wild type	NO_2^-	620
S1130	*narA*	NO_3^-	943
S1128	*narB*	NO_3^-	1,086
S1133	*narC*	NO_3^-	1,325
S1241	*narE*	NO_3^-	875
S1125	*anaA*	NO_3^-	1,250
S1125	*anaA*	NO_2^-	1,220

[a] The strains of the S series were derived from strain PAO by van Hartingsveldt et al. (15, 16) and were kindly supplied by A. Stouthamer. *nar* mutants lack the dissimilatory nitrate reductase. The *anaA* (*nirB*) mutant is affected in anaerobic growth, but has both nitrate and nitrite reductase activities (15, 16).

[b] These values were obtained after growth under low oxygen tension for 6.5 h, as described in the legend to Fig. 1.

TABLE 3. *Regulation of specific catabolic ornithine carbamoyltransferase activity by nutrient depletion during conditions of aerobiosis*

Growth medium	Growth phase[a]	Sp act (μmol of citrulline per h per mg of protein)
Glutamate (25 mM) + arginine (25 mM) + phosphate (0.2 mM)	Exponential	22
	Stationary	496
Pyruvate (8 mM) + NH_4^+ (25 mM)	Exponential	20
	Stationary	240

[a] Exponential phase, Activity measured during the five generations after inoculation; stationary phase, maximum activity reached 30 min after cessation of growth (cell density, 3×10^8 cells per ml).

The present study supports the idea that the deiminase pathway of *P. aeruginosa* is used to obtain energy under extreme conditions of nutrient depletion.

In full aerobiosis, the levels of arginine deiminase, catabolic ornithine carbamoyltransferase, and carbamate kinase were 1 to 2% of the levels obtained under conditions of induction. Depletion of oxygen as the terminal electron acceptor resulted in the strongest induction. Terminal electron acceptors themselves (oxygen, nitrate, nitrite) do not regulate the deiminase pathway directly. Thus, in the wild type, but not in *nar* mutants, nitrate partially inhibited the induction by oxygen limitation, indicating that a functional nitrate reductase is required for this inhibition. Depletion of the carbon and energy or phosphate source (conditions of stress) also leads to partial induction.

Glucose, fumarate, citrate, and pyruvate repressed the deiminase pathway during the shift from aerobiosis to anaerobiosis (Table 1). Glucose was most effective in this respect, although in *P. aeruginosa* tricarboxylic acid cycle intermediates are generally more effective in catabolite repression of inducible enzymes (10, 12, 17). The arginine decarboxylase pathway (9) was not induced by oxygen depletion (unpublished data).

Kight-Olliff and Fitzgerald (6) found that exogenous ATP inhibited the induction of alkylsulfatase in *P. aeruginosa*. In much the same way, ATP (6 mM) and UTP (6 mM) attenuated the induction of catabolic ornithine carbamoyltransferase under low oxygen tension, whereas AMP (6 mM) stimulated enzyme synthesis (unpublished data). Possibly, exogenous nucleotides modify the energy charge (2), which may be a critical signal in the induction of the arginine deiminase pathway. It has been shown that during aerobic growth of *P. aeruginosa* on a good carbon source (e.g., succinate) the energy charge is high (18). Depletion of an essential nutrient should result in a rapid decrease in the ATP level (18) and induction of the arginine deiminase pathway. In addition, ATP has been shown to be an allosteric inhibitor of catabolic ornithine carbamoyltransferase in *Pseudomonas putida* (14). This control may have an important function under conditions of noninduction.

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