JOURNAL OF BACTERIOLOGY, June 1982, p. 1085–1090
0021-9193/82/061085-06$02.00/0

Vol. 150, No. 3

# Control of Enzyme Synthesis in the Arginine Deiminase Pathway of *Streptococcus faecalis*

JEAN-PAUL SIMON,[1]* BRIGITTE WARGNIES,[2] AND VICTOR STALON[2]

*Institut de Recherches du Centre d'Enseignement et de Recherches des Industries Alimentaires et Chimiques, B-1070 Brussels, Belgium[1]; and Laboratoire de Microbiologie, Faculté des Sciences, Université Libre de Bruxelles, B-1070 Brussels, Belgium[2]*

Received 4 November 1981/Accepted 1 February 1982

The formation of the arginine deiminase pathway enzymes in *Streptococcus faecalis* ATCC 11700 was investigated. The addition of arginine to growing cells resulted in the coinduction of arginine deiminase (EC 3.5.3.6), ornithine carbamoyltransferase (EC 2.1.3.3), and carbamate kinase (EC 2.7.2.3). Growth on glucose-arginine or on glucose-fumarate-arginine produced a decrease in the specific activity of the arginine fermentation system. Aeration had a weak repressing effect on the arginine deiminase pathway enzymes in cells growing on arginine as the only added substrate. By contrast, depending on the growth phase, a marked repression of the pathway by oxygen was observed in cells growing on glucose-arginine. We hypothesize that, in *S. faecalis*, the ATP pool is an important signal in the regulation of the arginine deiminase pathway. Mutants unable to utilize arginine as an energy source, isolated from the wild type, exhibited four distinct phenotypes. In group I the three enzymes of the arginine deiminase pathway were present and probably affected in the arginine uptake system. Group II mutants had no detectable arginine deiminase, whereas group III mutants had low levels of ornithine carbamoyltransferase. Group IV mutants were defective for all three enzymes of the pathway.

Arginine is one of the substrates which, apart from carbohydrates, *Streptococcus faecalis* can use as an energy source for growth (2, 5). The degradation of arginine to ornithine and ATP has been demonstrated by Jones (10), and two enzymes of the pathway, ornithine carbamoyltransferase and carbamate kinase, have been isolated and characterized by Marshall and Cohen (12–14). Induction of the enzymes of the arginine deiminase pathway under oxygen limitation has been described in *Bacillus licheniformis* (3), *Pseudomonas aeruginosa* (16), and *Halobacteria* (9). It has been suggested that, in *P. aeruginosa*, conditions which lead to the formation of a decreased ATP pool result in the induction of the pathway, whereas, in contrast, high levels of the nucleotide triphosphate pool prevent this induction. The exponential phase of growth of *S. faecalis* is characterized by a continuous decrease of the ATP pool, the rate of decrease being a function of the energy source present in the medium (8).

In this paper, we investigate the regulation of the arginine deiminase pathway of *S. faecalis* by studying enzyme synthesis under a variety of growth conditions and the selection and the biochemical characterization of arginine-nonutilizing mutants (Aut$^-$ mutants).

## MATERIALS AND METHODS

**Media and growth conditions.** *S. faecalis* was grown either on a complex medium (medium A) or on an exhausted complex medium (medium B). The complex medium A buffered at pH 7.0 contained 10 g of yeast extract, 5 g of tryptone, 5 g of NaCl, 1.4 g of $KH_2PO_4$, 14.3 g of $Na_2HPO_4$, 1.7 g of $K_2SO_4$, 0.5 mg of $MgSO_4 \cdot 4H_2O$, and 5 mg of $FeCl_3$ per liter. Glucose, arginine, and fumarate were added at the desired concentrations. For aerobic cultures, 20 mg of hematin per liter was added to the medium. This basal medium supported a limited growth of *S. faecalis* in the absence of any added energy source. The amounts of reducing sugars, arginine (2 mM), and serine (3 mM) present in the medium account for this growth. At the beginning of the stationary growth phase arginine (<200 μM) and serine (<100 μM) were exhausted, whereas the other amino acids remained in excess. We have thus used a growth medium (medium B) which did not support growth of the organism unless an external energy source was added.

Medium B, exhausted in energy sources, was obtained by growing the cells aerobically to the stationary phase on medium A to deplete the energetic substrates initially present. Cells were discarded by centrifugation, and the medium was sterilized again by

filtration. On medium B glucose was a growth substrate only in aerobiosis, but not in anaerobiosis, unless the medium was supplemented with bicarbonate (20 mM) or arginine.

Cells were grown in a 2-liter Biolafitte microfermentor, and temperature and oxygen tension were monitored as described previously (16). Overnight cultures grown aerobically on medium B plus glucose were used as inoculum; the initial density in the fermentor was $10^7$ cells per ml. $Aut^-$ mutants were also grown overnight without agitation in sealed tubes.

**Isolation and characterization of $Aut^-$ mutants.** The $Aut^-$ mutants were isolated after treating the cells of *S. faecalis* ATCC 11700 by X-ray irradiation. Mutagenesis was carried out on cultures grown overnight in medium B supplemented with arginine as the energy source and suspended in mineral salt medium 154 (18). Eight milliliters of this suspension containing $10^8$ organisms per ml was irradiated in a petri dish with stirring. The total time of irradiation was chosen so that the number of survivors was 0.5%. The organisms were exposed to radiation from a type OEG 60 X-ray tube which was operated at 150 kV and 30 mA. The dose rate was 2.02 kilorads per min. The irradiated cells were then harvested by centrifugation and suspended on medium B containing arginine. After three or four generations, penicillin was added at a concentration of 200 U/ml. After 12 h, the remaining cells were washed and spread over petri plates containing medium B plus pyruvate. After growth, 2,000 colonies were obtained. Eighty of these strains were unable to grow on medium B supplemented with arginine as the energy source.

**Preparation of cell extract.** At time intervals, 50-ml samples of the culture were removed from the fermentor with a syringe and supplemented with chloramphenicol at 200 μg/ml. A 5-ml portion of the sample was first used to measure the optical density at 660 nm with a Gilford spectrophotometer and, after filtration through a membrane filter (0.45 μm; Millipore Corp., Bedford, Mass.), for the determination of the concentrations of glucose, arginine, citrulline, and ornithine. The remaining sample was centrifuged at 28,000 × *g* for 15 min. The cell pellet was washed with 0.9% (wt/vol) NaCl, suspended in 20 mM phosphate buffer (pH 7.5), and disrupted for 15 min in a Raytheon sonic oscillator at 10 kHz.

**Assay of enzyme activities in cell extracts.** Ornithine carbamoyltransferase was assayed by colorimetric determination of citrulline formed from carbamoylphosphate and ornithine as previously described (19). The arginine deiminase assay was essentially the same as previously described (18), except that 100 mM sodium citrate buffer (pH 6.0) was used. Carbamate kinase was assayed in the direction of carbamoylphosphate synthesis in a coupled assay containing ornithine and *Escherichia coli* ornithine carbamoyltransferase as described by Marshall and Cohen (13).

**Protein concentration.** Protein concentration was estimated by the method of Lowry et al. (11). Specific activities are expressed as the amount of enzyme which catalyzes the formation of 1 μmol of product per h per mg of protein.

**Analytical method.** The products of arginine catabolism were estimated in the clarified fluid with the amino acid analyzer as previously described (15). Glucose was estimated enzymatically by using the glucose oxidase-peroxidase test of Hoffmann-La Roche, Inc., Nutley, N.J.

## RESULTS

**Physiology of growth of *S. faecalis*.** Bauchop and Elsden have demonstrated that *S. faecalis* NCTC 6783 cannot use arginine as an energy source in the absence of glucose (2). In our experiments, *S. faecalis* ATCC 11700 did not require glucose since addition to the medium B of arginine as the sole energy source allowed growth of the organism. The increase in cell mass obtained anaerobically was directly proportional to the arginine concentration up to 50 mM. The corresponding ureido compounds (10%) and ornithine (90%) were the products of arginine utilization and appeared in the culture fluid.

**Regulation of ornithine carbamoyltransferase synthesis during anaerobic growth.** In our initial experiments, cells harvested in the stationary phase after anaerobic growth on medium A or B supplemented with arginine had high levels of ornithine carbamoyltransferase. When such cells were diluted into fresh A medium, the specific ornithine carbamoyltransferase activity dropped during the early stage of growth and increased again until the cells entered the stationary phase. Since induction can be best studied in cells with low initial specific activities, conditions were chosen to obtain stationary phase cells with relatively low activity. The lowest level of the enzymes of the arginine deiminase pathway was found in cells grown aerobically on glucose. These cells were routinely used as inoculum in derepression experiments.

When glucose-grown cells were diluted into medium B supplemented with 50 mM arginine, a 6-h lag was observed before growth resumed. During this time the synthesis of the arginine deiminase pathway occurred. When the enzymes reached a significant level, the culture began to grow with a generation time of 52 min. With the onset of exponential growth, the specific activities of the arginine deiminase pathway enzymes increased at a rate "quasi-parallel" to growth (data not shown). This increase of specific activities of all three enzymes of the pathway, arginine deiminase, catabolic ornithine carbamoyltransferase, and carbamate kinase, appeared coordinated (Fig. 1). In the following experiments, levels of catabolic ornithine carbamoyltransferase and arginine deiminase were determined under a variety of growth conditions, but carbamate kinase was not studied in such detail. An increase of arginine concentration up to 100 mM did not alter the differential rate of ornithine carbamoyltransferase synthesis (Table 1), but did enhance cellular growth and

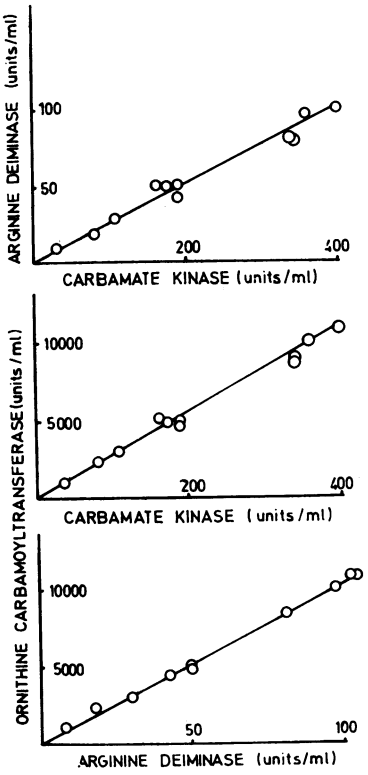


FIG. 1. Coordinate regulation of the enzymes of the arginine deiminase pathway in *S. faecalis* grown on medium A containing 50 mM arginine (see text).

TABLE 1. Ornithine carbamoyltransferase specific activity

| Medium | Culture | Energy sources | Ornithine carbamoyltransferase sp act[a] |
|---|---|---|---|
| B | Anaerobic | Arginine (100 mM) | 8,500 |
| B | | Arginine (50 mM) | 8,400 |
| A | | Arginine (50 mM) | 4,800 |
| A | | Glucose (50 mM) + arginine (50 mM) | 4,800 |
| B | | Glucose (50 mM) + arginine (50 mM) | 4,800 |
| A | | Glucose (50 mM) + fumarate (50 mM) + arginine (50 mM) | 3,400 |
| A | | Glucose (25 mM) | 800 |
| B | | Glucose (25 mM) | 30 |
| A | Aerobic | Arginine (50 mM) | 4,200 |
| A | | Glucose (50 mM) + arginine (50 mM) | 2,200 |
| A | | Glucose (25 mM) | 4 |

[a] As the specific activity of ornithine carbamoyltransferase increased continuously during the exponential phase of growth, the values given in the table are those for cells having reached the same cell density ($10^9$ cells per ml).

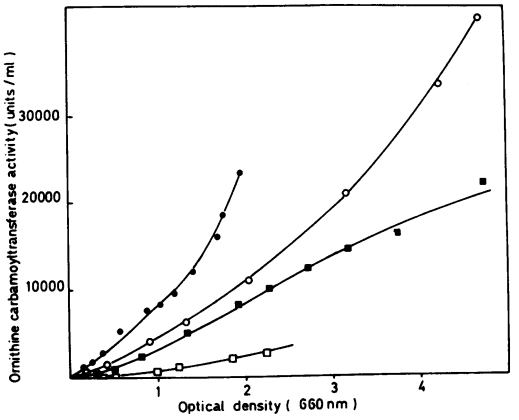


FIG. 2. Differential rate of ornithine carbamoyltransferase synthesis of *S. faecalis* grown in anaerobiosis in medium B (see text) supplemented with 100 mM arginine (●), 50 mM arginine plus glucose (○), or 50 mM arginine plus glucose and fumarate (■) or on medium A supplemented with 25 mM glucose (□).

consequently the specific activity of ornithine carbamoyltransferase entering the stationary phase. Addition to the medium of the fermentation products, namely, ornithine, citrulline, or $NH_4$, each at 25 mM, was without effect on the formation of ornithine carbamoyltransferase. The differential rate of ornithine carbamoyltransferase synthesis decreased when the cells were grown on medium B supplemented with glucose and arginine (50 mM each ) (Fig. 2). However, the pattern of enzyme formation depended on the growth phase (Fig. 3). Ornithine carbamoyltransferase activity reached a peak value during the exponential growth phase and decreased during the transitory growth phase occurring between the exponential and the stationary phase. The decrease in enzyme specific activity was not due to the removal of arginine since the growing organism fermented glucose and arginine simultaneously, and in the commencement of linear growth, each substrate concentration in the medium was decreased only by about 35% (Fig. 3). Addition of fumarate to glucose-arginine-grown cells also reduced the differential rate of ornithine carbamoyltransferase synthesis (Table 1). When cells were grown on medium B supplemented with glucose as the only substrate, low levels of ornithine carbamoyltransferase activity were observed. When medium A was used, some discrepancy was observed with the results obtained in medium B (Table 1). For instance, the differential rate of ornithine carbamoyltransferase synthesis on arginine was the same whether glucose was present or not. This discrepancy observed between medium A and B in ornithine carbamoyltransferase activity probably resulted from repression

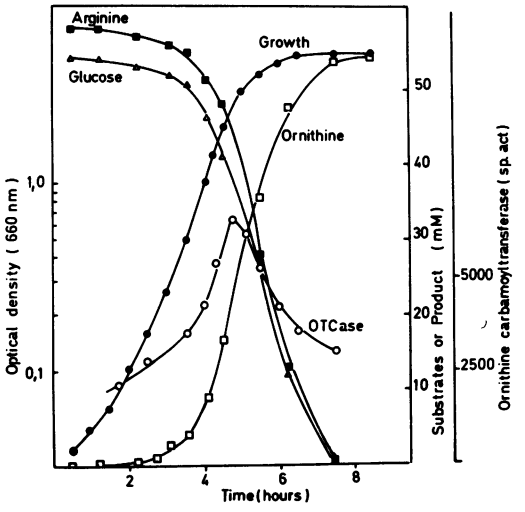


FIG. 3. Kinetics of ornithine carbamoyltransferase formation during growth on medium B supplemented with glucose and arginine. Symbols: optical density (●); glucose utilization (△); arginine utilization (■); ornithine excretion (□); ornithine carbamoyltransferase (OTCase) specific activity (○).

by carbohydrates present in medium A. On the contrary, the residual activity of ornithine carbamoyltransferase observed in medium A supplemented with glucose resulted from the presence of 2 mM arginine in this medium.

**Synthesis of ornithine carbamoyltransferase during aerobic growth.** Cells grown anaerobically or aerobically on the complex medium A and induced by arginine had approximately the same differential rate of ornithine carbamoyltransferase synthesis (Fig. 4). Supplementation with glucose considerably reduced the differential rate of ornithine carbamoyltransferase formation (Table 1). The inhibition of ornithine carbamoyltransferase synthesis resulting from the addition of glucose to an arginine culture was more effective than that produced under anaerobic conditions. However we observed (Fig. 5) that growth of *S. faecalis* in the medium containing glucose and arginine was characterized by the preferential utilization of glucose over arginine, in contrast with what was observed under anaerobic growth conditions (Fig. 3). The specific activity increased during the exponential growth, but with the arrest of the exponential growth, the specific activity declined although 90% of the initial arginine and glucose concentrations were still present. When glucose was exhausted from the medium, a rise in the specific activity was again observed, arginine being at this stage the only energy substrate present in the growth medium. When glucose was the only substrate added to the growth medium, low levels of ornithine carbamoyltransferase activity were observed (Table 1).

**Isolation of mutants unable to grow on arginine.** A total of 80 independent mutants of *S.*

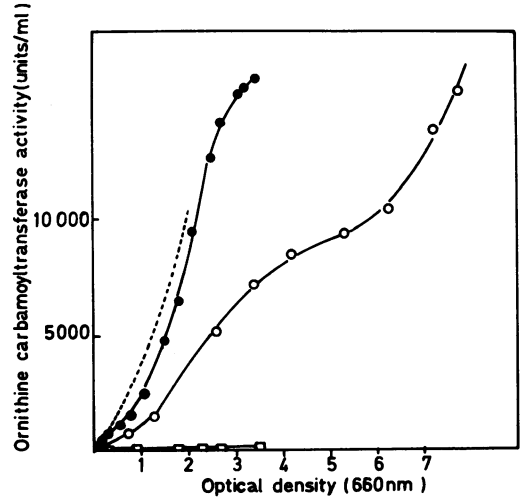


FIG. 4. Differential rate of ornithine carbamoyltransferase synthesis of *S. faecalis* grown in aerobiosis on medium A supplemented with 100 mM arginine (●), 50 mM arginine plus glucose (○), or 25 mM glucose (□). The dashed line represents the differential rate of ornithine carbamoyltransferase synthesis on arginine- and glucose-arginine-grown cells in anaerobiosis.

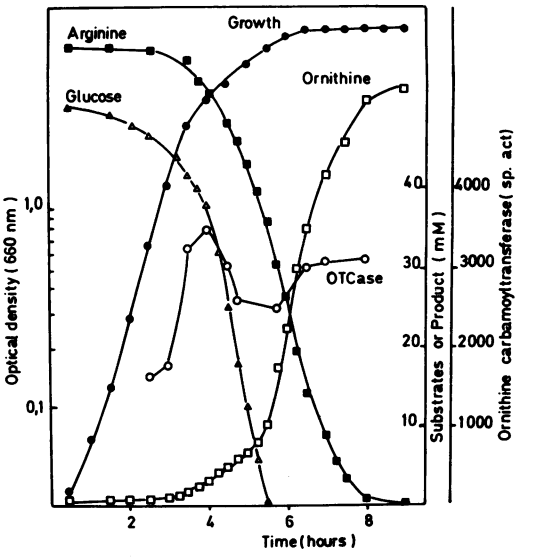


FIG. 5. Kinetics of ornithine carbamoyltransferase (OTCase) formation during growth in aerobiosis on medium A (see text) supplemented with glucose and arginine. Same symbols as in Fig. 3.

TABLE 2. Enzymes of the arginine deiminase pathway of *S. faecalis*[a]

| Strain[b] | Sp act | | |
|---|---|---|---|
| | Carbamate kinase | Ornithine carbamoyltransferase | Arginine deiminase |
| ATCC 11700 | 148 | 4,840 | 17 |
| Group I SW8 | 14 | 256 | 1.3 |
| SW13 | 30 | 750 | 3.2 |
| Group II SW19 | 93 | 2,760 | <0.2 |
| SW46 | 70 | 2,115 | <0.2 |
| Group III SW1 | 40 | 13 | 3.5 |
| SW74 | 23 | 24 | 0.8 |
| Group IV SW2 | <0.5 | 9 | <0.2 |
| SW25 | <0.5 | 7 | <0.2 |

[a] Cultures were grown anaerobically overnight in medium A containing 50 mM arginine.

[b] Results were selected for strains showing the largest variations of specific activities in each group.

*faecalis* unable to use arginine as growth substrate were isolated by X-ray irradiation and penicillin counterselection (see above). They have been assignated to four groups (Table 2). Except for the mutants of group III, all of these strains grown in medium A supplemented with 50 mM arginine were unable to use up arginine. Group III strains utilized some arginine (10%), which was recovered as citrulline excreted in the culture fluid. Group I mutants (SW13) had the three enzymes of the arginine deiminase pathway, but their levels were lower than those observed in the wild-type strain. We suspect that the altered regulation of the arginine deiminase pathway enzymes in these mutants could have been due to the impairment of arginine uptake, because arginine was not used in these cells.

Group II mutants (SW19) were found to be defective in arginine deiminase, whereas group III mutants (SW1) had low level of the catabolic ornithine carbamoyltransferase activity. Group IV mutants (SW25) had no detectable levels of arginine deiminase and carbamate kinase and a very low level of ornithine carbamoyltransferase activity (Table 2).

## DISCUSSION

In our experiments, arginine is an energy source for growth of *S. faecalis* ATCC 11700. The isolation of mutants altered in the arginine deiminase pathway and unable to grow on arginine as the only energy source strengthens the conclusion that ATP produced during arginine fermentation is used for the growth of the bacterium. Among the four groups of mutants, those of groups II and III are altered in either the activity of arginine deiminase or ornithine carbamoyltransferase. Mutants of group I, although having the three enzymes of the pathway, are unable to use up arginine. The obvious interpretation of this behavior is a defect in the uptake system of arginine. Mutants of group IV are clearly defective for all three enzymes of the pathway. The phenotype of these mutants suggests that they may have multiple mutations. Other possibilities are mutations in a regulatory gene essential for the transcription of an operon including the three genes of the pathway. A further possibility would be a polar mutation in the structural gene for one enzyme exerting polar effect on the expression of the other genes. Such polar effects have been recently described for the arginine deiminase pathway of *P. aeruginosa* (16a).

The formation of the arginine deiminase pathway in *S. faecalis* seems to be under the control of two processes: (i) induction by arginine, and (ii) repression of the induced synthesis by glucose, fumarate, and oxygen. The common product of aerobic metabolism, of fumarate reduction, and of the glycolytic pathway is ATP (4, 6, 7, 18). We consequently hypothesize that in *S. faecalis*, as in *B. licheniformis* (3) and *P. aeruginosa* (17), the ATP pool is an important signal controlling the regulation of the arginine deiminase pathway. Furthermore Forrest (8) has shown that during growth of *S. faecalis*, a fall in the ATP pool is always observed during the exponential growth phase, which in our experiments is always characterized by a logarithmic increase of the ornithine carbamoyltransferase specific activity.

The measurement of the ATP pool and the energy charge (1) in relation to the formation of the arginine deiminase pathway in *S. faecalis*, *P. aeruginosa*, and *B. licheniformis* is being planned for the future.

### ACKNOWLEDGMENTS

We are grateful to D. Haas for helpful discussion and to A. Piérard for reading the manuscript.

This work was supported by Fonds National de la Recherche Scientifique grant E 137. V.S. is research fellow of the Fonds National de la Recherche Scientifique.

### LITERATURE CITED

1. **Atkinson, D. E.** 1968. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry 7:4030–4034.
2. **Bauchop, T., and S. R. Elsden.** 1960. The growth of microorganisms in relation to their energy supply. J. Gen. Microb. 23:457–469.
3. **Broman, K., N. Lauwers, V. Stalon, and J. M. Wiame.** 1978. Oxygen and nitrate in the utilization by *Bacillus licheniformis* of the arginase and arginine deiminase routes of arginine catabolism and other factors affecting their syntheses. J. Bacteriol. 135:920–927.

4. **Bryan-Jones, D. G., and R. Wittenbury.** 1969. Haematin dependent oxidative phosphorylation in *Streptococcus faecalis*. J. Gen. Microbiol. **58**:247–260.
5. **Delbel, R. M.** 1964. Utilization of arginine as an energy source for the growth of Streptococcus faecalis. J. Bacteriol. **87**:988–992.
6. **Delbel, R. M., and H. J. Kvetas.** 1964. Fumarate reductase and its role in the diversion of glucose fermentation by *Streptococcus faecalis*. J. Bacteriol. **88**:858–864.
7. **Faust, P. J., and P. J. Vandemark.** 1970. Phosphorylation coupled to NADH oxidation with fumarate in *Streptococcus faecalis* 10CI. Arch. Biochem. Biophys. **137**:392–398.
8. **Forrest, W. W.** 1965. Adenosine triphosphate pool during the growth cycle of *Streptococcus faecalis*. J. Bacteriol. **90**:1013–1016.
9. **Hartmann, R., H. D. Sinckinger, and D. Oesterhelt.** 1980. Anaerobic growth of *Halobacteria*. Proc. Natl. Acad. Sci. U.S.A. **77**:3821–3825.
10. **Jones, M. E.** 1963. Carbamylphosphate. Science **140**:1373–1379.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. **193**:265–175.
12. **Marshall, M., and P. P. Cohen.** 1966. A kinetic study of the mechanism of crystalline carbamate kinase. J. Biol. Chem. **241**:4197–4208.
13. **Marshall, M., and P. P. Cohen.** 1972. Ornithine transcarbamylase from *Streptococcus faecalis* and bovine liver. J. Biol. Chem. **247**:1641–1682.
14. **Marshall, M., and P. P. Cohen.** 1977. Kinetics and equilibrium of the inactivation of ornithine transcarbamylase by pyridoxal 5'-phosphate. J. Biol. Chem. **252**:4267–4286.
15. **Mercenier, A., J. P. Simon, D. Haas, and V. Stalon.** 1980. Catabolism of L-arginine by *Pseudomonas aeruginosa*. J. Gen. Microbiol. **116**:381–389.
16. **Mercenier, A., J. P. Simon, C. Vander Wauven, D. Haas, and V. Stalon.** 1980. Regulation of enzyme synthesis in the arginine deiminase pathway of *Pseudomonas aeruginosa*. J. Bacteriol. **144**:159–163.

16a. **Mercenier, A., V. Stalon, J. P. Simon, and D. Haas.** 1982. Mapping of the arginine deiminase gene in *Pseudomonas aeruginosa*. J. Bacteriol. **149**:787–788.

17. **Ritchey, T. W., and H. W. Seeley.** 1976. Distribution of cytochrome-like respiration in *Streptococci*. J. Gen. Microbiol. **93**:195–203.
18. **Stalon, V., F. Ramos, A. Piérard, and J. M. Wiame.** 1967. The occurrence of catabolic and anabolic ornithine carbamoyltransferase in *Pseudomonas*. Biochim. Biophys. Acta **139**:91–97.
19. **Wargnies, B., N. Lauwers, and V. Stalon.** 1979. Structure and properties of the putrescine carbamoyltransferase of *Streptococcus faecalis*. Eur. J. Biochem. **101**:143–152.