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Biosynthesis and Metabolism of Arginine in Bacteria

RAYMOND CUNIN,[1] NICOLAS GLANSDORFF,[1,3]* ANDRÉ PIÉRARD,[2,3] AND VICTOR STALON[2]

Erfelijkheidsleer en Microbiologie, Vrije Universiteit Brussel,[1] Laboratoire de Microbiologie, Université Libre de Bruxelles,[2] and Institut de Recherches du Centre d'Enseignement et de Recherches des Industries Alimentaires et Chimiques,[3] B-1070 Brussels, Belgium

INTRODUCTION

The reasons why the biosynthesis and metabolism of arginine have been a focus of interest over the last 30 years reside for a large part in their higher degree of complexity than other pathways chosen as paradigms for studies in molecular physiology. This is evident in the extensive scattering of anabolic arginine genes displayed by most of the organisms investigated, in the occurrence of a biosynthetic branch point at the level of carbamoylphosphate (a precursor common to arginine and the pyrimidines), in the fact that ornithine or arginine is a potential precursor of polyamines, and in the impressive variety of degradative pathways that were found to occur, sometimes in the same organism. It was thus expected that the study of this system would lead to findings of general interest in the fields of enzymology, genetic control mechanisms, and metabolic physiology.

For historical reasons mainly, the present state of the art is very different whether one considers arginine biosynthesis in *Escherichia coli* (in particular, genetic control mechanisms) or other topics such as the nature and regulation of catabolic pathways. This situation is reflected in the different sections into which this review has been divided. Studies on the regulation of arginine biosynthesis in *E. coli* have seen the birth of the very concepts of repression (375) and regulons (202), as well as their extensive substantiation at the molecular level; this matter is therefore treated as a major section, after an account of the enzymological aspects of arginine biosynthesis and before a survey of genetic regulatory mechanisms in other bacteria. Data regarding catabolic pathways concern mainly the nature of the enzymatic steps involved and their physiological significance; despite the maze of information at hand, molecular genetic studies are still scarce. Therefore, we have considered it appropriate to treat each degradative pathway as an inclusive and separate section. Last but not least, studies on genes and enzymes involved in arginine metabolism have provided several observations of evolutionary interest, which are dealt with in the last section.

Polyamine biosynthesis is not considered in this review as a topic per se, but relevant information on this subject is considered in the subsection on the arginine decarboxylase pathway, which also deals with agmatine and putrescine catabolism. Several reviews on polyamines are available (342, 343; N. Glansdorff, *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger, ed., *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, in press).

Various aspects of arginine biosynthesis and degradation have been reviewed over the last 15 years (1, 27, 73, 75, 204, 270, 335, 382; Glansdorff, in press).

The role played by ornithine in the biosynthesis of iron-chelating hydroxamate siderophores (98) and the resulting interference exerted by arginine on some iron-requiring processes (176) are outside the scope of this review. This is also the case for the part taken by arginine, its precursors, and some polyamines in the biosynthesis of antibiotics (302).

* Corresponding author.

BIOSYNTHESIS OF ARGININE

Enzymatic Steps and Regulation of Metabolic Flow

From glutamate to arginine. The biosynthesis of arginine proceeds from glutamate in eight enzymatic steps (Fig. 1; Table 1). Five steps involving N-acetylated intermediates lead to ornithine. The conversion of ornithine to arginine requires three additional steps, the first of which involves carbamoylphosphate utilization. We deal in a separate section with the synthesis of this energy-rich metabolite, which participates also in the biosynthesis of pyrimidines.

The synthesis of ornithine, like that of proline, involves the activation and reduction of the 5-carboxyl group of glutamate. The product of this reaction is glutamate-5-semialdehyde, which in the nonacetylated form undergoes spontaneous cyclization, yielding the proline precursor 1-pyrroline-5-carboxylate. Acetylation of the 2-amino group of glutamate prevents this cyclization, thus keeping the two pathways separate (374, 376). After transamination onto the semialdehyde group to produce N^2-acetylornithine, the acetyl group is removed in a subsequent step, yielding ornithine. In procaryotes the pathway of arginine biosynthesis follows two alternative patterns which differ by the strategies used for the removal of the acetyl group and for the control of the metabolite flow along the pathway. *Enterobacteriaceae* and *Bacillaceae* use a linear pathway in which the formation of ornithine from N^2-acetylornithine is mediated by the hydrolytic enzyme acetylornithinase (286, 354, 378). In these organisms, *N*-acetylglutamate synthetase, the first enzyme of the pathway, is the target enzyme for feedback inhibition by arginine (383). In contrast, *Micrococcus glutamicus* and various other bacteria, including pseudomonads, cyanobacteria, photosynthetic bacteria, and *Thermus aquaticus* recycle the acetyl group by transacetylation of N^2-acetylornithine and glutamate (81, 140, 354, 356). In those organisms which utilize this energetically more economical version of arginine biosynthesis, *N*-acetylglutamate synthetase fulfills an anaplerotic function, and it is the second enzyme of the pathway, *N*-acetylglutamate 5-phosphotransferase, which is feedback inhibited by arginine (354, 357). This "more evolved" form of arginine biosynthesis is also present in eucaryotic organisms such as the fungi *Saccharomyces cerevisiae* and *Neurospora* spp. (79) and in green alga *Chlamydomonas* sp. (84). It has recently been found in several methanogenic bacteria (212).

Although the use of acetylated intermediates achieves an effective separation of arginine and proline biosyntheses, exchanges of metabolites between these pathways may be observed under particular conditions. *Pseudomonas aeruginosa* mutants with an early block in proline synthesis grow in the presence of high concentrations of L-ornithine (20 mM). This agrees with the observation that *N*-acetylornithine 5-aminotransferase of *P. aeruginosa* catalyzes the transamination of both N^2-acetylornithine and L-ornithine with

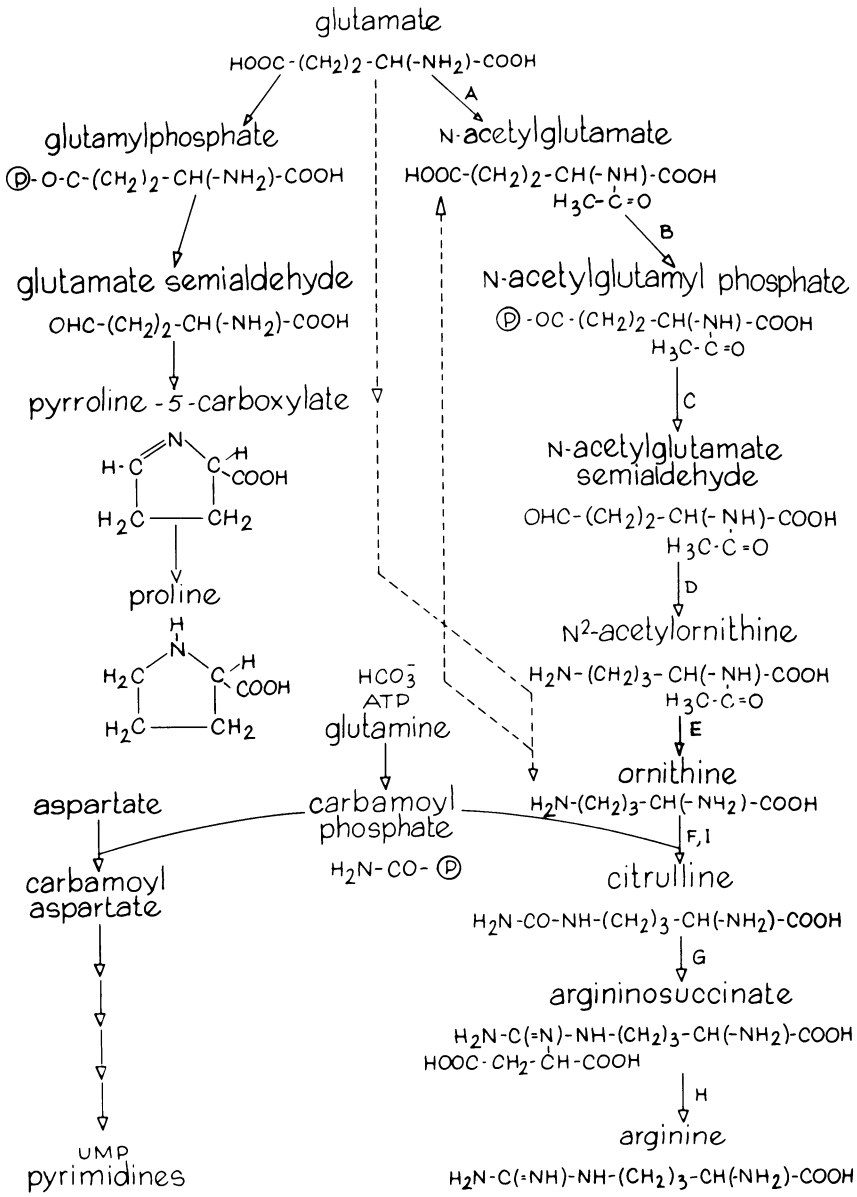

FIG. 1. Arginine biosynthesis and its relationship to the proline and pyrimidine pathways. Letters indicate the gene-enzyme relationship according to the terminology used for *E. coli*, *S. typhimurium*, *Pseudomonas* sp., and *B. subtilis*. The dashed line indicates the acetyl group recycling alternative to acetylornithinase.

2-oxoglutarate (371). Transamination of both substrates is also observed with the *E. coli* enzyme (36), and the conversion of *N*-acetylglutamate to proline by *E. coli* extracts has been shown to proceed via the transamination of ornithine (298). Nevertheless, *E. coli* mutants impaired in the early step of proline biosynthesis grow extremely slowly on ornithine, indicating that this transamination reaction has little physiological significance in vivo (36).

An interesting case of indirect suppression of proline auxotrophy has been reported, however, for *E. coli* and *Salmonella typhimurium* (151, 183). Suppression of *proA* and *proAB* auxotrophs in these organisms is provided by a block in gene *argD* encoding *N*-acetylornithine aminotransferase. The suppressed strains grow slowly on minimal medium, probably using another aminotransferase, and, due to lifting up of feedback inhibition and repression, accumulate *N*-acetylglutamate-5-semialdehyde. The latter intermediate is deacylated by the relatively unspecific *N*-acetylornithinase and converted to proline precursor glutamate-5-semialdehyde. Incorporation of arginine to the growth medium provokes feedback inhibition and represses *N*-acetylornithinase synthesis, thereby restoring the prolineless phenotype. In contrast to these observations, there has been no report of an arginine requirement being satisfied by intermediates of the proline pathway.

The properties of the enzymes involved in arginine biosynthesis in various procaryotes are summarized in the following paragraphs. The reactions they catalyze and their International Union of Biochemistry numbers are given in Table 1.

N-Acetylglutamate synthetase. The repression and feedback inhibition by arginine of the unstable *E. coli N*-

TABLE 1. Enzymes of arginine biosynthesis

Name	EC no.	Reactions catalyzed
N-Acetylglutamate synthetase	2.3.1.1	Acetyl-CoA + L-glutamate = CoA + *N*-acetyl-L-glutamate
N-Acetylglutamate 5-phosphotransferase	2.7.2.8	ATP + *N*-acetyl-L-glutamate = ADP + *N*-acetyl-L-glutamate 5-phosphate
N-Acetylglutamate 5-phosphate reductase	1.2.1.38	*N*-Acetyl-L-glutamate 5-phosphate + NADPH = *N*-acetyl-L-glutamate 5-semialdehyde + $NADP^+$ + orthophosphate
N-Acetylornithine aminotransferase	2.6.1.11	N^2-acetyl-L-ornithine + 2-oxoglutarate = *N*-acetyl-L-glutamate 5-semialdehyde + L-glutamate
N-Acetylornithinase	3.5.1.16	N^2-Acetyl-L-ornithine + H_2O = acetate + L-ornithine
Ornithine acetyltransferase	2.3.1.35	N^2-Acetyl-L-ornithine + L-glutamate = L-ornithine + *N*-acetyl-L-glutamate
OTCase	2.1.3.3	Carbamoylphosphate + L-ornithine = orthophosphate + L-citrulline
Argininosuccinate synthetase	6.3.4.5	ATP + L-citrulline + L-aspartate = AMP + pyrophosphate + L-argininosuccinate
Argininosuccinase	4.3.2.1	L-Argininosuccinate = fumarate + L-arginine
CPSase	6.3.5.5	2 ATP + L-glutamine + CO_2 + H_2O = 2 ADP + orthophosphate + L-glutamate + carbamoylphosphate

acetylglutamate synthetase was first demonstrated by Vyas and Maas (383), using resting cells. After the enzyme was stabilized in crude extracts and purified to homogeneity, it was possible to establish its main properties (194, 206). It is strongly inhibited by arginine (50% inhibition at a 0.02 mM concentration) but is unaffected by polyamines, in contrast to the *Pseudomonas* enzyme. It exists as several oligomeric forms made up of a single type of subunit of a molecular weight (MW) of 50,000. The apparent MW is dependent on protein concentration, suggesting that the regulation of the activity may involve ligand-induced changes of the state of oligomerization. The smallest aggregated form possessing catalytic activity is a trimer, whereas a hexamer is stabilized by the ligands L-arginine and *N*-acetyl-L-glutamate (206). Detailed kinetic data for the synthetase of *E. coli* have not been published but are available for the *S. typhimurium* enzyme, which is also strongly feedback inhibited by arginine (5). They indicate that the primary effect of the feedback inhibitor is to reduce the affinity of the enzyme for glutamate. The growth sensitivity to arginine of double *argD pro E. coli* mutants (see above) has been exploited to select feedback-insensitive mutants of the *argA* gene encoding *N*-acetylglutamate synthetase (95).

P. aeruginosa possesses the cyclic pathway of ornithine biosynthesis and uses *N*-acetylglutamate synthetase for an anaplerotic function (see the introduction to this section). Nevertheless, the use of a sensitive in vitro assay has shown that the synthetase is under multiple control by the products of the reaction and the end products of the pathway: synergistic inhibitions are exerted by *N*-acetylglutamate and polyamines on one side and by arginine, *N*-acetylglutamate, and coenzyme A (CoA) on the other (126, 127). *P. aeruginosa* is consequently a case where the flow of metabolites along the arginine pathway is regulated at the level of two consecutive steps. This same organism has also been found to contain an *N*-acetylglutamate deacetylase which is located in the periplasmic space and of which the physiological function remains unknown (110).

N-Acetylglutamate synthetase has been demonstrated in *Proteus mirabilis* (286) and in *Pseudomonas putida* (I. N. Chou and I. C. Gunsalus, Bacteriol. Proc., p. 231, 1971), but its regulatory properties have not been investigated in vitro. Its absence from *Legionella pneumophila* results in auxotrophy for arginine (345).

N-Acetylgutamate 5-phosphotransferase. The phosphorylation of *N*-acetylglutamic acid by adenosine triphosphate (ATP) in the presence of *N*-acetylglutamate 5-transferase is the target of feedback inhibition by arginine in those organisms which possess the cyclic pathway of ornithine synthesis. Therefore, this activity and its sensitivity to arginine have been investigated for a considerable number of procaryotes (23, 81, 140, 148, 195, 212, 354, 357). There are, however, few of these procaryotic phosphotransferases to have been studied in any detail. The feedback-insensitive enzyme of *E. coli*, encoded by gene *argB*, has been only partially purified and characterized (23, 379). Ultraviolet light-irradiated cells infected by λ *argECBH* transducing phages make a primary *argB* product of MW 29,000 (198).

The best-studied *N*-acetylglutamate 5-phosphotransferase is that of *P. aeruginosa*, which has been 90% purified by Haas and Leisinger (128). The enzyme is made of 29,000-MW subunits and exists in different states of aggregation depending on the ligands present. The MW is 230,000 in the presence of *N*-acetylglutamate, whereas smaller forms (minimal MW, 65,000) are observed in the presence of the feedback inhibitor L-arginine and Mg ATP, thus suggesting a ligand-directed association-dissociation equilibrium (128). In the absence of L-arginine, the saturation curves of both substrates are hyperbolic; when arginine is added, a sigmoid velocity response is observed with *N*-acetylglutamate, the "target" substrate, but not with Mg ATP. In contrast to *N*-acetylglutamate synthetase, the phosphotransferase is insensitive to the arginine degradation products agmatine, putrescine, and spermidine. The feedback inhibition of the first two enzymes of the pathway by arginine, as well as the critical role played by the intermediate metabolite *N*-acetylglutamate in this control, ensures a sensitive and immediate control of arginine synthesis in *P. aeruginosa* (129).

N-Acetylglutamate 5-semialdehyde reductase. Limited information is available concerning *N*-acetylglutamate 5-semialdehyde reductase, which in the biosynthetic direction catalyzes the reduction of *N*-acetylglutamylphosphate into the corresponding semialdehyde. The *E. coli* enzyme has been partially purified and some of its properties have been determined (380). Gel electrophoresis of the proteins synthesized by ultraviolet light-irradiated cells infected by λ *dargECBH* transducing phages have provided an estimation of 47,000 for the MW of the primary product of the *argC* gene encoding *N*-acetylglutamate semialdehyde reductase (198).

N-Acetylornithine aminotransferase. *N*-Acetylornithine aminotransferase, which catalyzes the reversible conversion of *N*-acetylglutamate-5-semialdehyde and glutamate to N^2-acetylornithine and 2-oxoglutarate, has been studied essentially for two bacteria, *E. coli* and *P. aeruginosa*.

E. coli W and K-12 have an amidotransaminase which is repressible by arginine (12). The enzyme is also active with glutamate-5-semialdehyde, thus yielding ornithine, although this activity probably has no physiological significance (36). By selecting for suppressors of *argD* mutations, mutants have been isolated in both strains that exhibit an arginine-inducible transaminase (22, 377; T. Eckhardt, Ph.D. thesis, ETH-Zürich, 1975). The inducible activity appears to result from the activation of *argM*, a cryptic gene unlinked to *argD*. The induction of *argM*, like the repression of *argD*, is mediated by the *argR* gene product (22). Both the inducible and the repressible *N*-acetylornithine aminotransferases have been purified to homogeneity (37, 105). The two enzymes differ by their MWs (119,000 for the wild-type transaminase, 61,000 for the inducible enzyme) and show no immunochemical cross-reactivity. Yet, both are made of 31,000-MW subunits and exhibit nearly identical tryptic digestion patterns, thus suggesting a common origin for these two genes. The evolutionary implications of such observations are discussed in another section of this review.

In *P. aeruginosa*, the catalytic properties of the transaminase and the regulation of its synthesis indicate that this enzyme is involved in the biosynthesis as well as the catabolism of L-arginine (371, 373). The enzyme, which has been purified to electrophoretic homogeneity, has an approximate MW of 110,000 and consists of two 55,000 subunits. It catalyzes the transamination of N^2-acetylornithine as well as that of L-ornithine with 2-oxoglutarate, the K_m for N^2-acetylornithine and ornithine being 1.1 mM and 10.0 mM, respectively (371). The transaminase is induced during growth on arginine as the only carbon and nitrogen souce and is repressed by various carbon sources (373). Recent results (D. Haas, personal communication; V. Stalon, unpublished observations) suggest that this enzyme is identical to succinylornithine aminotransferase, an enzyme of the succinyltransferase pathway of arginine catabolism (D. Vander Wauven, C. Legrain, and V. Stalon, manuscript in preparation). The properties of a mutant with an inactive acetylornithine aminotransferase indicate that this enzyme can be replaced by 4-aminobutyrate aminotransferase, an enzyme of putrescine catabolism (372).

In contrast to the previous organisms, *Klebsiella aerogenes* forms two separable acetylornithine aminotransferases (108). One is repressed by arginine and participates in its biosynthesis, whereas the second is induced by arginine and ornithine and functions in their catabolism. The inducible enzyme, which has been purified to near homogeneity, has an MW of 59,000 and exhibits activity with N^2-acetylornithine as well as with ornithine (107). The finding that this inducible aminotransferase has a fourfold lower K_m for succinylornithine than for N^2-acetylornithine suggests that it is also identical to succinylornithine aminotransferase (Vander Wauven et al., in preparation; see subsection, "Arginine Succinyltransferase Pathway").

N-Acetylornithinase. The hydrolytic enzyme *N*-acetylornithinase catalyzes the ornithine-yielding step of the linear pathway of arginine biosynthesis. It requires the presence of Co^{2+} ions and a thiol compound such as glutathion for its activity (378) and has been detected in a number of enterobacteria and bacilli (286, 354, 378). The best-characterized acetylornithinase is that of *E. coli*. It has been purified to homogeneity and, based on molecular sieving experiments, appears to be a monomer of MW 62,000 (J. Charlier, FEBS Meet. 1983, Brussels, Belgium, Abstr. SO-5; Charlier, personal communication). Acetylornithinase readily deacylates *N*-acetylglutamate semialdehyde, *N*-acetylarginine, *N*-acetylhistidine, *N*-acetylmethionine, and *N*-formylmethionine (26, 381). Advantage has been taken of this low substrate specificity of acetylornithinase and of its repression by arginine to select for fast-growing *argR* derivatives among *his* auxotrophs growing slowly on acetylhistidine (26), as well as *cis*-dominant mutations affecting *argECBH* expression (43, 67).

Ornithine acetyltransferase. Ornithine acetyltransferase catalyzes the transfer of the acetyl group of N^2-acetylornithine onto glutamate to yield ornithine and *N*-acetylglutamate; it is the key enzymatic step of the so-called cyclic pathway of ornithine synthesis (see the introduction to this section). It was first identified in *M. glutamicus* by Udaka and Kinoshita (356) and has since been demonstrated in a number of procaryotes including pseudomonads, photosynthetic bacteria, the thermophilic bacterium *T. aquaticus*, cyanobacteria, and methanogenic bacteria (81, 140, 212, 354). Yet none of these acetyltransferases appears to have been studied in any detail.

Some organisms have been found to possess both activities that achieve the conversion of N^2-acetylornithine to ornithine in vitro: ornithine acetyltransferase and acetylornithinase. In yeasts, mutants impaired in ornithine acetyltransferase grow slowly in the absence of arginine (220; F. Messenguy, personal communication), and the acetylornithinase measured in vitro can be ascribed to a metal-activated carboxypeptidase with little biosynthetic function (80). Such a carboxypeptidase is probably also responsible for the acetylornithinase activity of *T. aquaticus* (81). In *P. aeruginosa*, the two activities are separable by gel filtration, but their relative contributions to ornithine synthesis are unknown since no mutants lacking these activities have been isolated to date (125).

OTCase. Ornithine carbamoyltransferase (OTCase) serves two functions in arginine metabolism. In arginine biosynthesis it catalyzes the transfer of the carbamoyl moiety of carbamoylphosphate to the 5-amino group of ornithine, forming citrulline. In the catabolic arginine deiminase pathway (see the section on arginine catabolism), it mediates the thermodynamically less favored reverse reaction, the phosphorolysis of citrulline, yielding ornithine and carbamoylphosphate. Organisms that use both of these functions elaborate distinct anabolic and catabolic OTCases (186, 193, 338). The various anabolic OTCases exhibit similar structural, kinetic, and mechanistic properties. Most of them have MWs between 100,000 and 150,000; they are trimers of identical subunits, with MWs from 35,000 to 40,000 (193). This pattern of quaternary structure has been found for the anabolic OTCases of *E. coli* (189, 190), *S. typhimurium* (11), *P. putida* (339), *P. aeruginosa* (Stalon and Momin, unpublished data), and *Saccharomyces cerevisiae* (268). In *Aeromonas formicans* this basic trimeric structure (MW 125,000) exists in equilibrium with a heavier form (MW 420,000); the two forms do not differ by their kinetic constants (Momin and Stalon, unpublished data). A notable exception to the trimeric rule for the anabolic OTCases is the enzyme of *Bacillus subtilis* (243), which exists as a mixture of dimeric, tetrameric, and hexameric forms of a 44,000-MW subunit. Interestingly, OTCase is repressed by arginine in growing *B. subtilis* cells but is induced by arginine at the end of exponential growth. The physiological role of this induction by arginine is not known, but the enzyme synthesized under all conditions exhibits the same response towards antibodies against purified OTCase. This same enzyme is subject to inactivation followed by proteolytic degradation in sporulating cells (244).

In contrast to the anabolic OTCases, the catabolic OTCases usually show much more diverse structural features (100, 193). The evolutionary implications of such findings are discussed in a separate section.

The kinetic behaviors of the anabolic OTCases are usually consistent with ordered mechanisms in which carbamoylphosphate is the first substrate to bind and phosphate is the last product to be released or with random addition of the reactants with a preferred binding of carbamoylphosphate as the leading substrate (11, 190, 193, 243, 385). The patterns of inhibition of the anabolic OTCases of *E. coli* and *B. subtilis* by the bisubstrate analog *N*-δ-phosphono-acetyl-L-ornithine (PALO) are consistent with such mechanisms (243, 266). *E. coli* cells are impervious to PALO but are able to take up the oligopeptide gly-gly-PALO through the oligopeptide permease; the toxicity of PALO towards OTCase is expressed following its liberation by an intracellular peptidase (267). Such "illicit uptake" may have therapeutical implications as a means of driving into the cell a substance which is normally unable to penetrate into it (265).

All anabolic OTCases, except that of *Pseudomonas* spp., are able to catalyze both directions of the reaction (193). The functional irreversibility of the *Pseudomonas* enzyme results from the formation of a binary dead-end complex between the enzyme and citrulline that reduces the apparent maximum velocity (336). Such functional specialization may be important in organisms which use both directions of the OTCase reaction.

The position of OTCase half-way through the arginine pathway and the fact that the production of its second substrate, carbamoylphosphate, is in general highly regulated make a control of its activity unnecessary. A different situation is created, however, in organisms which possess an inducible arginase: after the addition of arginine and before the dilution of repressible OTCase by growth, an energy-wasteful urea cycle could operate that immediately degrades arginine formed in the biosynthetic pathway. In some yeasts and in *B. subtilis* this potential urea cycle is avoided by an arginine- and ornithine-dependent binding and inhibition of OTCase by arginase, called epi-arginasic regulation (149, 221). In *Agrobacterium tumefaciens* and several *Rhizobium* species in which arginase is inducible and OTCase is constitutive, the disadvantage of the simultaneous presence of both enzymes at a high level is corrected by feedback inhibition of OTCase by arginine (370; S. Vissers et al., manuscript in preparation). OTCase inhibition in *S. typhimurium* and *P. putida* occurs at relatively high arginine concentrations and has probably less physiological importance (11, 336).

The structure of *E. coli* OTCase has been studied in particular detail. Interestingly, *E. coli* K-12 carries two genes for OTCase, *argF* and *argI*, both repressible by arginine (118); their products interact to form a family of four trimeric isoenzymes which can be separated by ion-exchange chromatography (189). The *F* and *I* isoenzymes have similar kinetic parameters but differ in their thermostabilities (191). Only gene *argI* or its equivalent can be found in *E. coli* B and W or in other *Enterobacteriaceae* (189, 191). The occurrence of hybrid *F-I* isoenzymes has suggested that these genes originate from the duplication of a common ancestral gene. In addition, the adjacency of *argI* and *pyrBI* (152) encoding aspartate carbamoyltransferases as well as the structural and catalytic similarities observed between the two carbamoyltransferases has led to a belief that these enzymes arose from an analogous genetic event (189). These hypotheses are supported by the recent determination of the primary structure of these genes and are considered in more detail under "Evolutionary Considerations."

Argininosuccinate synthetase. Argininosuccinate synthetase, which catalyzes the conversion of citrulline, aspartate, and ATP into argininosuccinate, has not been well studied for procaryotes but its yeast counterpart has been characterized in some detail; it is a tetramer of identical 49,000-MW subunits (137). From denaturing gel electrophoresis of extracts from minicells producing a plasmid-encoded argininosuccinate synthetase, the *E. coli* enzyme appears to consist of a basic polypeptide of similar MW, 48,000 (239).

Argininosuccinase. Little is known of the bacterial argininosuccinases, which hydrolyze argininosuccinate into arginine and fumarate. Extracts of ultraviolet light-irradiated *E. coli* cells infected with an *argH* transducing phage contain a polypeptide of 55,000 MW (198), which may be similar to the 50,000 subunit of the better-characterized tetrameric mammalian argininosuccinase (295).

Biosynthesis of carbamoylphosphate. Three types of organization may be distinguished among procaryotes with regard to the biosynthesis of carbamoylphosphate. The first type corresponds to organisms which use a single enzyme to produce carbamoylphosphate required for arginine and pyrimidine biosynthesis. It is widely distributed among gram-negative bacteria and, in particular, *Enterobacteriaceae*. This group of organisms is best illustrated by *E. coli*. In this organism, one-step mutants can be isolated which lack a single glutamine-dependent carbamoylphosphate synthetase that is regulated in a manner consistent with its dual metabolic function: cumulative repression by arginine and pyrimidine and modulation of the activity by effectors belonging to the two pathways which utilize carbamoylphosphate (18, 269, 271, 273). A low carbamate kinase activity detectable in *E. coli* and in some other enteric bacteria is due to a constitutive acetate kinase and probably has no biosynthetic significance (71, 165, 273, 346). Carbamate kinase itself, previously believed to play a role in carbamoylphosphate synthesis (162), is now assigned an essentially catabolic function as an enzyme of the arginine deiminase pathway (see the section on arginine catabolism).

A second type of organization is represented by *B. subtilis*, which elaborates two independently regulated carbamoylphosphate synthetases: one is repressed by arginine; the second is repressed and feedback inhibited by pyrimidines (262). No other gram-positive bacterium has been studied in comparable detail. Thus it is not known whether *B. subtilis* is unique among procaryotes in displaying this type of organization or whether it is representative of a wider group of organisms.

Still another way of forming carbamoylphosphate is used by *Lactobacillus leichmanii*, and possibly other lactic bacteria, which seems to lack any carbamoylphosphate synthetase activity but possesses the arginine deiminase pathway of arginine catabolism. Tracer studies suggest that this organism derives the carbamoylphosphate required or pyrimidine biosynthesis from the degradation of arginine (146).

This section is devoted to a summary and discussion of the properties and control of the activity of *E. coli* and other well-characterized bacterial carbamoylphosphate synthetases.

Enterobacterial CPSases. The single carbamoylphosphate synthetases (CPSases) from *E. coli* (for review see reference 214), *S. typhimurium* (1, 8), and *Serratia marcescens* (71) are well characterized and appear very similar in structural, catalytic, and regulatory properties. All three are subject to

cumulative repression by arginine and uracil (1, 71, 273) as well as to activation by ornithine and feedback inhibition by uridine monophosphate (UMP) (1, 18, 71, 269, 271).

E. coli CPSase is the most thoroughly studied of these enzymes. It catalyzes a reaction in which the amide group of glutamine, a bicarbonate ion, and two ATPs are used for the synthesis of carbamoylphosphate (16). Ammonia, a lower-affinity nitrogen donor for the reaction in vitro, probably plays no physiological role (165, 166). Studies with highly purified CPSase preparations have shown that the reaction proceeds in four steps: (i) ATP-dependent activation of carbon dioxide under the form of enzyme-bound carbonate-phosphate anhydride; (ii) reaction of this intermediate with glutamine; (iii) transfer of the amide nitrogen group of glutamine to activated carbon dioxide to form enzyme-bound carbamate; (iv) use of a second ATP to phosphorylate carbamate and liberation of carbamoylphosphate (17).

The observation that a chloroketone analog of glutamine inactivates the glutamine binding site of CPSase without affecting the ammonia-dependent activity has provided clear evidence that these two nitrogen donors react with separate binding sites on the enzyme (177). In addition, relatively mild conditions were shown to promote the reversible dissociation of the enzyme into two nonidentical subunits: a small subunit (MW 42,000) displaying glutaminase activity in vitro and a large subunit (MW 130,000) which catalyzes the synthesis of carbamoylphosphate from ammonia, bicarbonate, and ATP (348). These two subunits are encoded by the adjacent genes *carA* and *carB* of *E. coli* (219) which are organized in an operon oriented from A to B (70, 114). The complete sequences of these genes, as determined recently, provide MWs of 41,270 and 117,710 for their respective protein products (250, 277). The equivalent proteins in *S. typhimurium* are encoded by the locus *pyrA* (8). No information is available concerning their genetic determinants in *Serratia marcescens*.

The mechanisms of glutamine utilization for carbamoylphosphate synthesis is similar to that proposed for other glutamine amidotransferases (133). It involves binding of glutamine to the small subunit and transfer of its amide nitrogen group to an ammonia binding site on the large subunit (280, 348, 349). Extensive interactions between the light and heavy subunits of the enzyme appear to facilitate its catalytic function. For example, the isolated light subunit exhibits a much lower affinity for glutamine than does the native enzyme (349). In addition, the binding of the chloroketone analog of glutamine to the light subunit decreases the apparent K_m for ammonia (280).

CPSase is a highly regulated enzyme which is inhibited by UMP, and to a lesser extent by other uridylic nucleotides, and activated by ornithine; its activity is also enhanced by ammonium ions, by inosine monophosphate, and by various other purine nucleotides (18, 269, 271). In *E. coli* the heavy subunit of CPSase, which carries all of the catalytic functions of the enzyme except the hydrolysis of glutamine, clearly also bears the binding sites for the allosteric effectors (348, 349). In *S. typhimurium*, however, the purified heavy subunit is but weakly inhibited by UMP though addition of the light subunit restores normal sensitivity towards this nucleotide; it is not known whether this effect reflects interactions between the two subunits or the presence of an UMP binding site on the light subunit. The antagonistic effects of UMP and ornithine provide an elegant manner to regulate the supply of carbamoylphosphate according to the needs of the two pathways which utilize it (269). Indeed the cellular concentration of ornithine varies in inverse ratio to arginine owing to feedback inhibition of *N*-acetylglutamate synthetase by arginine (269). The activation of CPSase by inosine monophosphate and the other purine nucleotides achieves a balance between the relative rates of purine and pyrimidine biosynthesis (18).

CPSase, which displays a sigmoidal ATP saturation curve (18), exists in at least three conformational states: a form which has no affinity for the substrate ATP and is stabilized by UMP; in equilibrium with this form, a second form, which is stabilized by ornithine and inosine monophosphate and has affinity for ATP; and a third form, which is catalytically active and into which the second one is converted by ATP binding (14, 15). The monomer undergoes a reversible self-association in the presence of the allosteric effectors (349) which is not related to the regulatory and catalytic properties of the enzyme (13).

The ATP molecules which participate in two different steps of the reaction catalyzed by CPSase bind at separate sites on the heavy subunit (39, 285). Nyunoya and Lusty (250), after determining the sequence of *carB*, have observed an homology between the two halves of the sequence, thus suggesting that this gene arose from the duplication of an ancestral gene. They have also proposed that the allosteric behavior of the enzyme reflects interactions between distinct folding domains corresponding to the two half sequences, each possibly carrying one of the ATP binding sites.

Due to the complexity of its catalytic and regulatory properties, it is not surprising that a variety of non-biauxotrophic phenotypes were observed among mutants affected in *E. coli* CPSase (3, 219, 271). The first of these mutants to be studied were uracil sensitive (271; G. Leclercq, Ph.D. thesis, Université Libre de Bruxelles, Brussels, Belgium, 1971). The mutant enzyme of strain P678M1, for example, displays an increased K_m for ATP in such a manner that in the presence of UMP, and even in the presence of ornithine, the activity is too low to support growth. The apparent affinity for ATP is so low in the absence of ornithine that the mutant is also partially sensitive to arginine.

Such phenotypes have also been obtained in *S. typhimurium*. Uracil sensitivity in a cold-sensitive mutant results from the increased inhibition of the enzyme by UMP at low temperature (2). Other phenotypes are of particular interest since they seem to impair complex enzyme interactions and protein maturation mechanisms. They are discussed in the section devoted to control of gene expression in *S. typhimurium* (9, 10).

P. aeruginosa CPSase. The single CPSase of *P. aeruginosa* shows much similarity with its enteric counterpart (7). The enzyme uses either glutamine (K_m 0.15 mM) or NH_3 (K_m 17 mM) as the nitrogen donor. It has an MW of 165,000 and is composed of two nonidentical subunits (MW 44,000 and 122,000) which are probably equivalent to the *carA* and *carB* products of *E. coli* (7). Mutations resulting in double auxotrophy for arginine and uracil have been obtained (125, 200). They map in a single *car* locus in which it has not been possible to distinguish the equivalent to genes *carA* and *carB* (125; C. Vander Wauven, personal communication). The CPSase of *P. aeruginosa* is subject to partial cumulative repression by arginine and pyrimidines; it is feedback inhibited by UMP and activated by ornithine and N^2-acetylornithine (7; C. Vander Wauven, unpublished data). The effect of N^2-acetylornithine can be considered an index of arginine limitation under conditions in which ornithine is rapidly catabolized (7).

A particularity of *Pseudomonas* spp. is the simultaneous

presence of CPSase and carbamate kinase under conditions in which the arginine deiminase pathway is induced. This would allow the operation of a wasteful cycle resulting in the loss of one molecule of ATP per molecule of carbamoylphosphate processed. The energetic disadvantage of such a situation is probably minimized owing to the inhibition by ATP of the activity of carbamate kinase (6). The catabolic OTCase is also inhibited by ATP, thus avoiding an exaggerate conversion of carbamoylphosphate into citrulline (C. Legrain and V. Stalon, unpublished observations).

B. subtilis CPSase. B. subtilis, in contrast to the gram-negative bacteria discussed in the previous paragraph, has two CPSases with different physical and catalytic properties and with different sensitivities to feedback inhibition and to repression (262). Already suggested by the isolation of mutant strains which display arginine-sensitive or uracil-sensitive phenotypes (283), the existence of these two enzymes has been confirmed by the characterization of the CPSases present in extracts of wild-type and mutant strains (262). CPSase A, absent from uracil-sensitive strains, has an MW of 200,000 and is repressible by arginine. It is insensitive to metabolites of the arginine or pyrimidine pathways. CPSase P (MW 90,000 to 100,000), which is repressible by uracil, is lacking in arginine-sensitive strains; it is severely inhibited by uridine nucleotides and activated by PRPP and guanosine monophosphate. Both isoenzymes utilize glutamine and, less efficiently, ammonia and require K^+ ions for activity and stability (262). This previously unrecognized fact may explain earlier difficulties encountered in characterizing CPSases of this organism (150, 283).

B. subtilis is unique among procaryotes studied to date in possessing two CPSases and in this respect displays a striking similarity to fungi and, in particular, yeasts (185). It will be interesting to study the regulation of carbamoylphosphate synthesis in other gram-positive bacteria.

Other procaryotic CPSases. Glutamine-dependent CPsase was detected in various other procaryotes including *Neisseria gonorrhoeae* (240), *T. aquaticus* (81), and at least two cyanobacteria, *Synechoccus* sp. strain PCC 6301 and *Synechocystis* sp. strain PCC 6308 (A. Feller, D. Vander Wauven, V. Stalon, and A. Piérard, unpublished data). The use of ammonia for carbamoylphosphate synthesis, possibly through carbamate kinase, has been reported in *Ectothiorhodospira shaposhnikovii* and *Rhodospirillum rubrum* (178).

Evidence based on the isolation of double auxotrophs for arginine and pyrimidine suggests the existence of a single CPSase in *Proteus mirabilis* (288) and *N. gonorrhoeae* (240). A nonrepressible glutamine-dependent CPSase was demonstrated in the latter organism.

Control of Gene Expression in *E. coli*

The genes coding for the arginine-biosynthetic enzymes of *E. coli* are scattered around the chromosome (19). They nevertheless constitute a physiological unit controlled by a single repressor, i.e., a regulon (202). As the extent of the repression response varies considerably from enzyme to enzyme, it has been proposed very early that the repressor (the *argR* gene product) interacts with different though related operators (120, 201). The genes coding for CPSase are particularly interesting in this respect since the synthesis of this enzyme is cumulatively repressed by arginine and the pyrimidines (273).

From the outset, regulation of arginine biosynthesis in *E. coli* presented a paradox: while in strains K-12 and W the enzymes of the pathway are repressible by arginine, they are induced by this amino acid in strain B. In a first unitary account of regulation in the arginine system, Jacoby and Gorini (154) showed by genetic studies that *E. coli* B possessed a regulatory gene ($argR_B$) allelic to $argR_{K\text{-}12}$. In *E. coli* B excess arginine appeared to favor a form of the repressor displaying reduced affinity for the operator sites; in a lower concentration range arginine actually repressed enzyme synthesis. A single amino acid substitution caused the B repressor to behave like its K-12 counterpart. In diploids combining B and K-12 alleles (or wild-type and mutant ones) the allele conferring the lowest enzyme level was always dominant (164).

Establishing the level of control (transcriptional or post-transcriptional) required the construction of transducing phages and plasmid vectors to be used in deoxyribonucleic acid-ribonucleic acid (DNA-RNA) hybridization experiments and as in vitro templates. The relevant experimental strategies have been reviewed in detail by Cunin (73) and Glansdorff (in press), who also described the steps taken more recently to clone and sequence individual *arg* genes. Only the final results of these investigations are discussed here. From the information available emerges a simple model for transcriptional regulation; in essence, it bears out the early proposal. Interestingly, no attenuation control appears to be involved, a feature shared by lysine and methionine biosynthesis (91, 308, 341). The possibility of a second site control remains a pending question, discussed in the subsection, "Levels of control."

Genetic organization. Of all *E. coli arg* genes, *argECBH* constitutes the only cluster. It is a divergent operon (97, 153) the two arms of which, *argE* and *argCBH*, are transcribed from two promoters facing each other over an internal operator region (97 and below). *argF* is peculiar to *E. coli* K-12; this strain appears unique among the *Enterobacteriaceae* in having this second gene for OTCase in addition to *argI*. *argM*, which codes for a cryptic, inducible acetylornithine transaminase, lies in the *ilvA-argECBH* region (see above, *N*-acetylornithine aminotransferase) but is not localized accurately (301; Eckhardt, Ph.D. thesis). The genes for CPSase, *carA*, and *carB* (formerly *pyrA*) form an operon controlled from adjacent tandem promoters (40, 277) respectively controlled by arginine and the pyrimidines (277; see below).

It is not yet known whether *argS*, the structural gene for arginyl-transfer RNA (tRNA) synthetase, belongs to the regulon. The genes involved in arginine transport apparently do not (see section on transport). The possible significance of the genetic layout of the *arg* regulon is discussed in "Evolutionary Considerations." Mutants with mutations in *arg* genes can be obtained by a variety of methods, including forward selections for *arg* and *car* auxotrophs. Several ways of selecting for *argR* (derepressed) mutants have also been described (Glansdorff, in press).

Levels of control. Estimates of pulse-labeled RNA hybridizing with the DNA of phages transducing the *argECBH* genes indicated that the major part of the control of those genes was transcriptional (74, 182). This conclusion was extended in vitro and shown to apply as well to *argA*, *-F*, and *-I* and *car* (199, 272, 303, 318, 319). However, only in the case of *argECBH* and *carAB* operons were the measurements sufficiently precise to assess whether repression of DNA transcription and repression of enzyme synthesis paralleled each other over the whole range of gene expression. Using purified single-strands of λ *argECBH* transduc-

ing phages as hybridization probes, it could be shown that the rate of *argE* or *argCBH* messenger RNA (mRNA) synthesis varied three- to fourfold less than the cognate enzyme activities (74). Others (182) confirmed the general trend of these data by measuring the bulk of *argECBH* hybridizable mRNA and were able to reproduce the phenomenon in vitro (401). In contrast, estimates of *carAB* mRNA and CPSase activities paralleled each other closely (272).

The relative excess of *argECBH* RNA observed in repression revived the notion (209) that the control of *arg* genes might be at least partly translational. Since restricting the arginine supply increased three- to fourfold the chemical half-life of *argECBH* hybridizable RNA, it was proposed that derepression stabilized *arg* mRNA (210). However, since bulk RNA proved to be affected in the same way (182), no specific *argR*-mediated effect on *argECBH* mRNA stability could be regarded as an explanation for the mRNA-enzyme discrepancy. The possibility of such an effect was also critically investigated in the case of OTCase mRNA, with negative results (131).

The discrepancy could have been explained in the frame of Lavallé's kinetic analysis of enzyme repression (188). As addition of arginine to a growing culture provoked a temporary stagnation of acetylornithinase and OTCase synthesis followed by slow recovery, the author proposed that an immediate effect of arginine addition, via the repressor and another protein X of the regulon, was to freeze mRNA in an inactive state, until the quantity of this protein became diluted by growth. If, as a result of this interaction, the efficiency of translation had remained relatively lower in repression than in derepression, a discrepancy between the respective evolutions of enzyme-specific activities and of mRNA levels would have been observed. However, later experiments indicated that the temporary stagnation of enzyme synthesis probably resulted from enzyme decay rather than translation arrest (272).

When the phenomenon of attenuation was discovered, another explanation was proposed: if repressor inhibited initiation of DNA transcription less efficiently than arginyl-tRNA attenuated a leader transcript, repressed cells would contain a relatively higher proportion of unproductive leader mRNA (74). In keeping with this proposal, hybridizable *arg* RNA from repressed cells appeared to be shorter than that from derepressed cells (182). However, when evidence for a possible *argECBH* leader RNA was investigated, both in vitro and in vivo, none could be found; clearly, repression did not lead to preferential transcription of operator-proximal sequences (34). Last but not least, when the DNA sequences of the *argECBH*, *argF*, *argI*, and *carAB* genes were determined, no evidence for attenuation control could be found either (see below).

It remains possible that the discrepancy is due to a greater instability or lesser abundance of distal portions of mRNA beyond the region abutting the control region, but it is unclear how this phenomenon would be controlled by repression. In our opinion another possibility is worth investigating: it was shown recently (see below, "Structure of control regions and the repression response") that a secondary *argE* promoter (*argEp2*) located in *argC* accounts for a substantial fraction of the *argE* messenger produced in repression (75; J. Piette, Ph.D. thesis, University of Brussels, Brussels, Belgium 1983). The *argEp2*-initiated mRNA overlaps *argCBH* mRNA by 194 base pairs; this provides ample opportunity to form RNA duplexes that would presumably not engage in translation and therefore account for the discrepancy. This explanation is of course ad hoc for *argECBH*, but it must be recalled that no enzyme-RNA discrepancy was observed in the case of *carAB* (272).

Besides *argR*-mediated control of DNA transcription, the nucleotide ppGpp, chemical messenger of the stringent response, influences the expression of *arg* genes, but in a positive way (182, 402). These references correct an earlier report (391). The phenomenon has been investigated in detail in vitro with *argECBH*. The data indicate that the main effect of ppGpp is exerted at the level of translation. Zidwick et al. (402) at first suggested that ppGpp acted at some early step in the coupling of translation to transcription, but more recent experiments from the same laboratory (M. G. Williams, Ph.D. thesis, University of Minneapolis, Minneapolis, Minn., 1985) indicate that initiation of translation is not involved. Rather, it appears the ppGpp is necessary for correct translation into functional enzymes of *arg* mRNA accumulated during starvation. Elaborating on ideas proposed by O'Farrell (252) and Menninger et al. (215) (see also reference 111), the author suggests that ppGpp would indirectly reduce the frequency of translation errors. Actual mistranslation of *arg* mRNA into faulty enzymes by *relA* cells remains to be demonstrated, however.

Positive effects of ppGpp have also been noted for *argA* and *argI*, as for other amino acid-biosynthetic genes (111, 170, 353). On the other hand, the expression of *carAB*, like that of *pyrB* (40, 353), is partially inhibited by ppGpp in keeping with the presence of a guanine-plus-cytosine-rich stretch downstream from the pyridimine-specific promoter P1 (see Fig. 2). Via this mechanism the stringent response would thus involve substantial turnoff of the synthesis of a class of RNA precursors.

Formation of active repressor. For some time after the discovery of the *argR* gene by the isolation of mutants resistant to canavanine (120, 201), it remained debated whether the corepressor was arginine or arginyl-tRNA. Early observations showed that the intensity of repression did not parallel the level of arginyl tRNA charging (139) and that the charging profiles of the five isoacceptor $tRNA^{arg}$ were indistinguishable in repressed and derepressed strains (57, 196). Most significantly, in *argS* mutants impaired in their charging ability, OTCase synthesis not only remained repressible but proceeded at a lower rate than in the wild type (139). This suggested that the partial block led to intracellular accumulation of arginine which, directly or indirectly, but not via tRNA, activated the aporepressor. This view was challenged by (i) the isolation of mutants claimed to be altered in both their arginyl-tRNA synthetase activity and in the repressibility of arginine biosynthetic enzymes (42) and (ii) experiments establishing a link between derepression and inhibition of the synthetase by endogenously accumulated argininosuccinate (42). These experiments could not be confirmed or were shown to involve artifacts (61). Later on, the nature of the corepressor could be directly assessed in vitro. Arginine and partially purified repressor free of arginyl-tRNA synthetase were shown to repress *argECBH* and *carAB* transcription (77, 199). Moreover, in unpublished experiments from this laboratory, G. Bény was unable to observe any effect of $tRNA^{Arg}$ or of the synthetase on *argCBH* transcription in vitro. L-Ornithine, L-citrulline, and D-arginine tested in vitro did not affect transcription of *argECBH* or the synthesis of enzymes E and H (401); these results are in keeping with previous in vivo experiments (68) and correct an earlier report (119). Present evidence thus demonstrates that arginine acts as corepressor in vitro and strongly suggests that it

plays this role in vivo to the exclusion of other molecules. Binding of arginine to the repressor has not yet been demonstrated, however.

Attempts at purifying the aporepressor have remained confined to the K-12 strain. The *argR* locus appears to contain one functional unit (164). Using differential labeling, Udaka (355) purified a protein of MW 45,000. Using in vitro protein synthesis as an assay system (358), Kelker and co-workers (171) obtained up to 70-fold-purified preparations which were useful in several studies on the mechanism of repression in coupled or purified systems. It is not yet known whether active repressor consists of elements other than the *argR* product and arginine. From in vitro transcription experiments Udaka (personal communication) suggests that an additional protein which binds arginine may be involved in repression.

No totally pure repressor is available presently, but the cloning and sequencing of the *argR* gene led Eckhardt (personal communication) to calculate an MW of 17,000 for the *argR* monomer. The number of repressor molecules per cell was calculated from coupled translation-transcription assays combining S30 extracts from $argR^+$ and *argR* cells in various proportions. The estimates vary from 40 to 200 (199); this range is compatible with the moderate escape from repression observed in cells carrying multiple copies of *arg* and *car* genes (69, 70, 117).

Autogenous regulation of *argR* expression (see next section) provides the cell with a means to increase the number of repressor molecules when the concentration of arginine is low; this could explain why the enzymes are still 80 to 90% repressed in minimal medium. A similar situation obtains in tryptophan biosynthesis (172).

The properties inferred from the genetic analysis of the *E. coli* B repressor (see above introduction to the arginine regulon) would make it a particularly interesting subject of study. Now that its more "classical" K-12 allelic form has been cloned, cloning the $argR_B$ gene should be straightforward.

Structure of control regions and the repression response. The search for the postulated target sites of the *argR* product required the isolation of *cis*-dominant mutations altering repression. As the *arg* genes did not constitute an operon, several ad hoc approaches were deveoped, including selection for the suppression of polar effects in the *argECBH* operon (97), relief from repression of streptomycin-induced suppression of *argI* or *argC* nonsense mutations (153, 154), selection for derivatives of *his* auxotrophs able to utilize acetylhistidine (a substrate for acetylornithinase) in the presence of a source of arginine (43, 67), and acquisition of the ability to use citrulline as a source of carbamoylphosphate for pyrimidines by backwards working OTCase (*argF* and *argI* O^c mutants; 192). Last but not least, in a more general approach, in vivo engineered *arg-lacZ* fusions have been used (33), thanks to the genetic tools developed by Casadaban and Cohen (52).

Published DNA sequences are available for the control regions of genes *argECBH* (274), *argF* (229, 275), *argI* (276), and *carAB* (40, 277). Complete sequences have been published for *argF* (366), *argI* (32), and *carAB* (250, 277); they are mentioned in the section, "Evolutionary Considerations." Promoter sites have been assigned by polymerase binding, fingerprint experiments, S1 mapping, and sequencing mutations in the case of *argECBH* (274), by the two latter approaches for *carAB* (40, 277; D. Charlier and M. Roovers, unpublished experiments from this laboratory), and by S1 mapping alone for *argF* and *argI* (276). *argD* (cloned in collaboration with M. Riley [301]) and *argG* (cloned in collaboration with Y. Nakamura [239]) have been partly sequenced in our laboratory; tentative assignments for the promoters of these two genes were made by S1 mapping. Most of this information is summarized in Fig. 2. *argA* and *argM* have not yet been investigated.

A common feature of all control regions investigated so far (including those of *argD* and *-G* [not shown here]) is the presence of the so-called "*arg* box": an 18-base-pair-long, partly conserved, and distinctly palindromic sequence. Sequencing operator mutations in *argECBH* (75, 274), *argF* (366), and *carAB* (Charlier and Roovers, unpublished experiments) established the role of the *arg* box as the basic operator module of the *arg* regulon. A consensus derived from the comparison of these sequences is given in Fig. 3.

In all cases, at least one *arg* box overlaps the promoter. This suggests that steric hindrance between repressor and RNA polymerase is the basis of the repression response, in keeping with the results of in vitro experiments varying the order of addition of S30 extracts from $argR^+$ and *argR* strains (319). *argG*, however, presents an additional putative operator site 110 nucleotides from the main start of transcription; the role of this site is unknown.

There is a crude correlation between the number of *arg* boxes present and the amplitude of the repression-derepression response. On the one hand, relatively low values (10 to 15) obtain for one-box genes such as *argH* in the *sup102* deletion mutant (Fig. 2) and the autogenously regulated *argR* gene. On the other hand, *argD*, *carAB* (both with one "good" box and a more degenerate one), and genes with two good boxes, i.e., *argE* (the contribution of the secondary promoter *argEp2* excluded [see below]), *argCBH*, *argF*, and *argI*, display increasingly higher values (Table 2). The tightest repression response characterizes *argF* and *argI*, where the overlap with the promoter is the most extensive of all and the extent of dyad symmetry is the highest. Since the *lac* operon is expressed over an even wider range and displays a relatively weak overlap, we would assume the extent of symmetry to be the dominant factor. A comparison of the *trpEDCBA*, *trpR*, and *aroH* promoter-operator regions leads to the same conclusion (392). Moreover, constitutive mutations of *carAB* and *argF* were found to alter two positions which are both highly conserved and involved in symmetry (Fig. 2). Symmetry is not the only factor involved, however; as in the *lac* operon the tightness of the response must also depend on the actual composition of the box since *argECBH* O^c mutants may result from substitutions altering a position in the right-hand part of the box, a position which is neither conserved nor involved in symmetry (75). Since the right-hand half of the consensus *arg* box is the lesser conserved one, it is conceivable that variations in this sequence play an important role in determining the respective affinities of the different operator sites for the repressor.

Considering the above correlation and the fact that all pairs of *arg* boxes are separated by the same number of nucleotides (75), it is possible that repressor molecules bind at adjacent boxes in a cooperative way. In support of this suggestion it can be mentioned that a deletion of 1 base pair between the two *argECBH* boxes determines partial constitutivity, as though efficient repression required the two boxes to remain in register (75, 274).

A notable characteristic of all of the above control regions is the lack of typical attenuation features. Both *argCBH*- and *argG*-coding sequences are, however, preceded on the mRNA by leader sequences. Their functions, if any, are

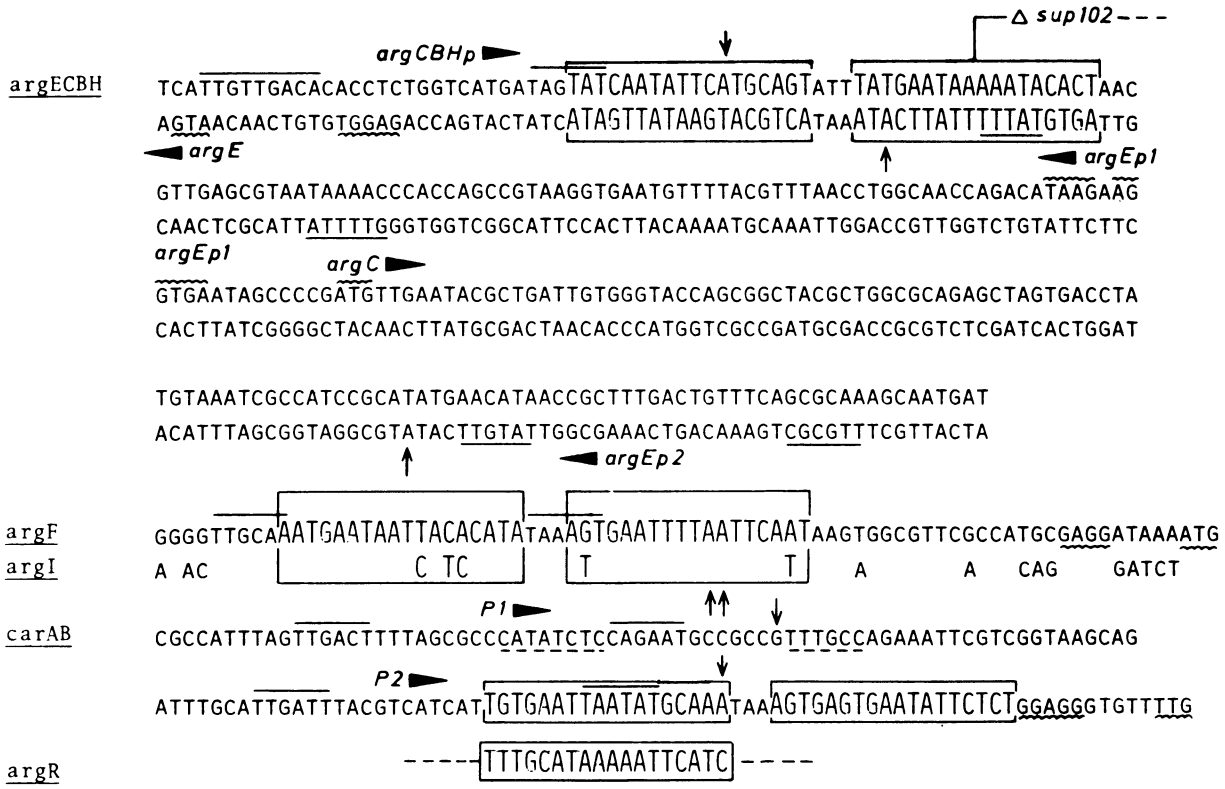

FIG. 2. Structure of control regions for genes involved in arginine (*arg*) and carbamoylphosphate (*car*) synthesis in *E. coli*. Gene-enzyme relationship as in Fig. 1. *argR* codes for the aporepressor. In the case of *argI* only the nucleotides differing from the *argF* sequence are mentioned. (Promoters) Straight lines above the sequence (or under it for *argEp1* and *argEp2*) refer to −35 and −10 regions; polarity is indicated by a horizontal arrowhead and each transcription startpoint is indicated by a vertical arrow. Dashed lines in *carAB* indicate homology with the promoter of *pyrBI*, the aspartate carbamoyltransferase operon. (Operators) Operator modules (*arg* boxes) are framed. (Translation signals) Wavy lines indicate sequence complementarities to the 3'OH extremity of the 16S RNA and translational start codons.

unknown; *aroH* presents a similar case (404). *argD*, *-E*, *-F*, and *-I* have only very short leader sequences.

Bipolar *argECBH* operon and divergent transcription. The *argECBH* promoter-operator complex is the most complex one of the whole regulon. The basic features of this region, two promoters facing each other over a central and common regulatory region, were predicted on formal genetic grounds (97) before the divergent character of the *argE* and *argCBH* transcripts was established by RNA-DNA hybridization experiments (258, 284). At that time the latter type of approach had already shown that the biotin operon was divergently transcribed (121); later work (see discussion in reference 274) established how closely the respective organizations of the two gene clusters actually resemble each other.

Our most recent data (75; Piette, Ph.D. thesis) show that the *argECBH* control region is even more complex than originally thought since a secondary promoter, *argEp2*, has been discovered in the *argC* gene. In repression, this weak but not negligible promoter is responsible for a wave of *argE* transcription which can be detected by S1 mapping experiments. Insertion of bacteriophage Mu between *argEp2* and the main *argE* promoter interrupts expression from *argEp2* and brings the repressed level of acetylornithinase to about half the wild-type level (A. Boyen, unpublished experiments from this laboratory). This background transcription from *argEp2* therefore explains why the derepression-repression response for acetylornithinase is only 15 to 20, while it is 50 to 70 for argininosuccinase; even though *argEp* and *argCBHp* share an operator region and they overlap roughly to the same extent.

It is of interest that mRNA is actually transcribed from *argEp2* under conditions in which the repressor must be binding to DNA about 110 nucleotides downstream; moreover, abundant and partly constitutive synthesis of *argE* mRNA results from mutations improving the Pribnow box of *argEp2* (75) or from insertion of the outward promoter of IS*3* 180 nucleotides upstream from the *argEp1* transcription start (59). It therefore appears that, in vivo at least, actual steric hindrance between repressor and polymerase binding is necessary to achieve efficient repression. Early experiments on *trp-lac* fusions already suggested this (299), and the P1 promoter of the *carAB* operon (see below) provides another case.

S1 mapping experiments (75; Piette, Ph.D. thesis) indicate that transcription from *argEp2* in the wild type or in *argEp2* "up" mutants vanishes when *argCBH* is derepressed; on the other hand, it increases in *argCBHp* "down" mutants. The mechanism of this interference is not known, but several hypotheses may be considered: hybridization and subsequent degradation of complementary mRNA molecules respectively initiated at *argEp2* and *argCBHp*; and collisions between polymerases or unavailability of *argEp2* when *argC* is actively transcribed from *argCBHp*.

The promoter for *argCBH* is at first sight atypical since the distance separating the most proximal one of two possible −10 regions from a perfect fit to the −35 consensus sequence, TTGACA, is only 15 nucleotides. It is possible that

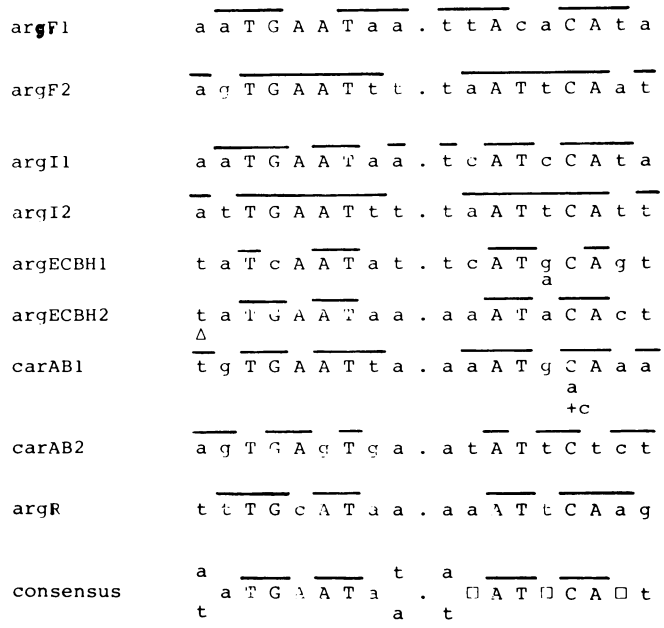

FIG. 3. Comparison and consensus between *arg* and *car* operator modules (*arg* boxes). Nucleotide substitutions, deletions (△), or additions (+) observed in operator mutants are indicated below the line. The size of the letter refers to the degree of conservation of the cognate nucleotide. Hyphenated symmetries are overlined.

the TTG triplet immediately preceding the previous sequence plays a crucial role in polymerase-promoter interactions.

A particularly interesting type of mutation simultaneously increases the efficiency of *argE* translation and decreases the efficiency of *argCBH* transcription (41). The mutations fall in the −10 region of the *argCBH* promoter which, on the other strand, corresponds to a site located between the 5′ end of *argE* mRNA and the sequence binding to 16S RNA. Since the mutations, in contrast with many other cases, do not appear to affect possible secondary structures, it is conceivable that they concern nucleotides involved in direct interaction with ribosomal proteins or initiation factors, in line with an earlier suggestion (312).

***carAB* operon and cumulative repression.** Among various procaryotic genes known to be transcribed from multiple promoters, the *carAB* operon stands out as a case of particularly clear-cut significance from the physiological point of view. Expression from P1, the upstream promoter (Fig. 2), is specifically prevented in the presence of excess pyrimidines, while P2 is repressed by arginine (277). The two mechanism concur in establishing the regulatory pattern previously defined as "cumulative repression" (271, 273).

Several problems remain to be solved, however. First, the mechanism by which pyrimidines control P1 is not known. Nothing in the sequence would point to attenuation (in contrast with *pyrB*), but some sequence similarities between the control region of *pyrB* and P1 suggest a common regulatory mechanism, possibly of the negative type (40, 277). In *Salmonella* spp. (see below), unlinked mutations, some of which affect RNA polymerase, alter the rate of expression of *carAB* and *pyr* genes. Their equivalent has not yet been isolated in *E. coli*.

A second question is how P1, the upstream promoter, is expressed when the arginine repressor is bound to the *arg* boxes overlapping P2. This situation is not without precedent since (i) the *trp* promoter is only partly repressed by downstream bound *lac* repressor (299) and (ii) transcription proceeds from *argEp2*, in particular in up mutants, when repression strongly inhibits *argEp1* and *argCBHp* (see previous section). It is possible, however, that P1-bound polymerase actively destabilizes P2-bound repressor. The two sites are close enough to each other to make such an interaction conceivable.

A last question also focuses attention on the possibility of interactions between transcriptional and regulatory factors bound at the two abutting promoters: unpublished S1 mapping experiments (Piette, Ph.D. thesis) show that, in the presence of pyrimidines and arginine, repression at P2 (the arginine-specific promoter) becomes more intense than with arginine alone. Does a pyrimidine-specific regulatory factor somehow tighten arginine-mediated control or is the observed repression enhancement an appearance resulting from suppression of a positive effect of P1-bound polymerase on transcription initiation at P2? Experiments are in progress to distinguish between those alternatives.

Of two putative and adjacent translational start codons, UUG and AUU, exclusively the former one is used to initiate *carA* translation (388). The efficiency of *carA* translation is about 40% that of the *lacZ* gene (388); UUG is therefore no insuperable obstacle to efficient translation. Since, however, the UUG codon was recently shown to be an intrinsically much weaker translational start than AUG (297), the relatively high yield of *carA* translation may be attributed to context effects as already suggested (388).

The complete sequence of the *carAB* operon is known (250, 277). The two genes are separated by a short intercistronic space. While the structure of *carA* and *carB* raises no obvious problem, it is worth noting that some *carB* nonsense mutants, as yet unsequenced, display surprisingly strong "antipolar" effects on the expression of *carA* (114). The actual cause of these effects remains to be established.

Arginyl-tRNA synthetase. It is not yet known whether arginyl-tRNA synthetase, the *argS* gene product, is part of the *arg* regulon, though *argS* has been shown to become permanently derepressed during arginine starvation (230, 242). Since attenuation does not appear to be used for control of the *arg* genes investigated so far, it will be particularly interesting to see whether this pattern holds for *argS* as well; derepression of *phe*-tRNA synthetase has been correlated with attenuation (351). As for several other synthetases, the specific activity of the *argS* protein is positively correlated with the growth rate, in approximate balance with tRNA, elongation factors, and ribosomes (241).

TABLE 2. Repression response in the arginine regulon of *E. coli* K-12 (sequenced genes)

Transcription unit	Approximate repression-derepression ratio[a]	No. of *arg* boxes
argR	10	1
argD	20	2
carAB	35[b]	2
argE	40[c]	2
argCBH	60	2
argF	180	2
argI	350	2

[a] Ratio between specific activities of the cognate enzymes respectively assayed in genetically derepressed cells (no arginine added) and wild-type cells (100 μg of arginine per ml added).

[b] Uracil (100 μg/ml) added to the cultures.

[c] Contribution of secondary *argEp2* promoter excluded.

Other properties of the enzyme have been reviewed recently (Glansdorff, in press).

Concluding remarks. As the group of genes controlled by the *lex* repressor, the arginine regulon of *E. coli* is one of the best-known examples of a scattered set of structural genes repressed by a unique aprepressor, recognizing with varying efficiency the members of a family of related operators. No other type of regulatory interaction could be demonstrated up to now within the system. The occurrence of long leader sequences with no apparent function in *argC* and *argG* is puzzling and requires further investigations; *argA* and *-M* remain to be sequenced.

This "classical" type of gene regulation is now understood in its major characteristics, though repressor binding remains to be studied in vitro. Several mechanistic problems which were uncovered en route remain to be solved: the *argECBH* RNA-enzyme discrepancy, the exact mechanism of interference between the convergent *argCBH* and *argEp2* promoters, the P1-P2 interactions and the pyrimidine target in *carAB*, and the regulatory mechanisms controlling the synthesis of the permease (see below) and the arginyl-tRNA synthetase. It is also clear that further studies with the arginine system will contribute to put to the test new ideas on the effect of ppGpp on translational fidelity, while the *carAB* operon will provide an interesting system for studying the mechanism of translation initiation at the atypical codon UUG. Last but not least, several features of the *arg* regulon are interesting to discuss from the evolutionary point of view; this will be done in the last section of this review.

Control of Enzyme Synthesis in Other Procaryotes

On the basis of the information available, procaryotes may be divided roughly into two categories according to the pattern of regulation which prevails for their arginine biosynthesis (73, 354). Bacteria which possess an ornithine acetyltransferase and recycle the acetyl group from acetylornithine to glutamate appear to rely mostly on feedback inhibition by arginine of the second enzyme, acetylglutamate phosphotransferase, to control the pathway. In *Enterobacteriaceae* and *Bacillaceae*, which do not recycle the acetyl group, arginine represses the synthesis of all or most of the enzymes and inhibits acetylglutamate synthetase, the first enzyme of the pathway. It is not known whether this correlation has biological significance or if it will resist further screening. After a general survey of these two groups, we shall deal separately with *Pseudomonas* spp., *S. typhimurium*, and *B. subtilis*, organisms in which arginine biosynthesis has been or is being studied systematically.

Bacteria lacking or showing limited repression control. With the exception of *Pseudomonas* spp., the current state of knowledge of the regulation of arginine biosynthesis in bacteria of this group is still fragmentary, and caution should be exercised when generalizing from observations relative to only a few or even a single enzyme of the pathway.

In blue-green algae (cyanobacteria) and in several photosynthetic bacteria, feedback inhibition of acetylglutamate phosphotransferase appears as the sole regulating mechanism controlling biosynthesis (51, 140, 141). Similarly, repression control is apparently absent in *Corynebacterium glutamicum* and other micrococci (354, 397), *N. gonorrheae* (in which only CPSase has been investigated [240]), *Agrobacterium tumefaciens*, and *Rhizobium* spp. (in which only OTCase was studied [370; Vissers et al., manuscript in preparation]).

In the extreme thermophile *T. aquaticus*, synthesis of ornithine acetyltransferase is repressed sevenfold by arginine (81).

Data are now available about microbes from the third kingdom of life, archaebacteria, which are considered equidistant from procaryotes and eucaryotes (106). Methanogenic bacteria possess an ornithine acetyltransferase and appear to lack acetylglutamate synthase (212). Acetylglutamate phosphotransferase is feedback inhibited by arginine. No repression by arginine is observed (212).

***Pseudomonas* spp.** In *Pseudomonas* spp., ornithine is produced from acetylornithine by an ornithine acetyltransferase. It is not known whether the acetylornithinase activity also found in *P. aeruginosa* has biological significance (see above, "Enzymatic Steps and Regulation of Metabolic Flow").

Carbamoylphosphate for both pyrimidine and arginine biosyntheses is made by a single CPSase. Mutations in the corresponding gene (*cpa*) result in a double auxotrophy (125). The genes for six of the nine biosynthetic enzymes and for CPSase have been mapped in *P. aeruginosa*. They are scattered on the chromosome (125).

Control of biosynthesis is achieved mainly through feedback regulation of the activity of the first two enzymes of the pathway (126, 129, 354). However, regulation of synthesis is observed for two enzymes in *P. aeruginosa*: anabolic OTCase (aOTCase) and acetylornithine aminotransferase, respectively repressed and induced by arginine (148, 371). The latter enzyme, which is now referred to as succinylornithine aminotransferase (see "Arginine Succinyltransferase Pathway") is involved both in biosynthesis, in which its basal uninduced level allows synthesis of acetylornithine, and in degradation of arginine. A similar regulation of enzyme synthesis has been observed for *P. putida*, in which, in addition, the synthesis of acetylglutamate phosphotransferase or of the dehydrogenase or both seems repressible too (63, 279). Repression of aOTCase synthesis by arginine and induction of acetylornithine aminotransferase seem to be mediated by a common regulatory element since a linear relationship is observed between the reciprocal of the acetylornithine aminotransferase specific activity and that of aOTCase (373). Ornithine also induces the aminotransferase and represses aOTCase biosynthesis, which raises the question, in view of the key situation of ornithine in the metabolism, of which molecule (arginine or ornithine) is the true effector of the genetic regulation. The syntheses of aOTCase and aminotransferase are also modulated by carbon sources (148, 373). The synthesis of the aminotransferase is repressed by citrate, a better carbon source than arginine, while aOTCase synthesis is derepressed in the inverse ratio (148, 373). It was suggested (148) that the carbon source regulation functions indirectly through the arginine regulator, possibly via an inhibition of arginine uptake by citrate and other good carbon sources. This hypothesis is supported by the characterization of mutants able to derive proline from ornithine (via the aminotransferase) in the presence of citrate (CIT^r mutants [373]). A single mutational event alters dramatically the citrate regulation of both the aminotransferase and aOTCase and abolishes arginine-specific regulation. Carbon source regulation does not involve cyclic adenosine monophosphate (cAMP). In *P. putida* too, a considerable difference in the amplitude of control of aOTCase and aminotransferase synthesis is observed between cells grown on arginine as sole carbon or carbon and nitrogen source and cells grown in the presence of a more readily utilizable substrate (279). This

difference is correlated to a modulation of arginine uptake by catabolite repression (279).

CPSase shows cumulative repression by arginine and pyrimidines (7). The observed amplitude of repression appears considerably smaller than in *E. coli*, ranging from 40% (7) to 75% (Vander Wauven and Piérard, unpublished data). Here too, the contribution of arginine to repression appears to be modulated by carbon source regulation (Vander Wauven and Piérard, unpublished data), which may account to some extent for the low repressibility of CPSase synthesis.

Bacteria with extensive repression control. In several *Bacillus* species, OTCase synthesis is repressed at least 10-fold by arginine (354; see below). A comparable repression is observed in the enteric bacteria *K. aerogenes* and *Serratia marcescens* (354). The amplitude of repression by arginine is quite small in *Proteus* spp. (O. W. Prozesky, M.D. thesis, University of Pretoria, Pretoria, South Africa 1968) (287).

As in *E. coli*, several genes of the biosynthetic pathway are clustered in *Proteus morganii*, *Proteus rutgeri*, and *Proteus mirabilis*, but in addition to *argECBH*, they include *argG* (*argECBGH*) (Prozesky, M.D. thesis) (289). A similar clustering is found in *Serratia marcescens* (207). A cluster identical to that of *E. coli* (*ECBH*) is present in *Salmonella* spp. (309), *Providentia stuartii* (298), and *K. pneumoniae* (208). In *B. subtilis* also, extensive clustering is found, but it involves other arginine genes (235; see below).

S. typhimurium. The genetic organization of the biosynthetic genes is similar to that of *E. coli* (309). There is only one gene encoding OTCase, the equivalent of *argI*, a situation which prevails among *E. coli* strains other than K-12. Repression by arginine is mediated by the product of a regulatory gene, *argR* (174). Repressibility ratios are of the same order of magnitude as those observed in *E. coli* (175). The regulatory regions (operator sites) must be homologous to their *E. coli* counterparts since almost normal repression of the cognate genes is restored by the *E. coli* repressor in hybrid F′ *argR*$^+$ *E. coli*/*argR*::Tn*10* *S. tyhpimurium* merodiploids (112). The much more limited repression by the *E. coli* repressor reported earlier (175) for merodiploids constructed with an *argR* mutant of *Salmonella* sp. selected as a suppressor of proline auxotrophy in the presence of arginine (174) has been suggested to be the consequence of the formation of interspecific hybrid repressor molecules.

The best-studied enzymes are OTCase and CPSase. Antibodies raised against *Salmonella* OTCase cross-react with the *E. coli* enzyme (11) and vice-versa (100). As in *E. coli*, the situation of CPSase at the interface of the pyrimidine and arginine pathways is mirrored in the array of enzymatic and genetic controls to which it is submitted.

Genetic control takes the shape of a cumulative repression by arginine and by the pyrimidines via a cytosine compound (1). Whether this compound acts through the arginine repressor or through a pyrimidine-specific aporepressor remains a matter of conjecture, but the interaction of a cytosine compound with the arginine repressor might explain the increased repression of OTCase when both cytosine and arginine are present in excess amounts (173). In *E. coli*, cumulative repression is achieved through the semi-independent regulation of transcription initiation at two tandem promoters (see above, "Control of Gene Expression in *E. coli*").

Several mutant phenotypes originate from the interaction between arginine and pyrimidine biosyntheses. Similarly to what is observed in *E. coli*, bradytrophic *pyrH* mutants exhibit a partial arginine requirement due to the increase of carbamoylphosphate consumption by the derepressed pyrimidine pathway, compounded by a limited carbamoylphosphate synthesis resulting from the inhibition of CPSase by the increased UMP concentration (158). Some missense mutations at the *pyrA* locus, which do not abolish catalytic activity, cause uracil sensitivity, arginine sensitivity (ARS) (9) or pseudo-arginine auxotrophy (AUX) (10).

A particular uracil-sensitive phenotype (Use$^-$) seems to result from the hyperrepression of *pyrA* as well as of other genes by uracil (47). Interestingly, the pyrimidine genes affected are those presumed to be regulated by a cytosine compound, which suggests that the *use* locus could encode a product acting pleiotropically as a regulatory element (47). This regulatory effect might, however, be an indirect one. Indeed, a fluorouracil-resistant mutant, in which two of the uridine-repressible *pyr* genes (*pyrB*, *pyrE*) are highly derepressed and *pyrA* is slightly derepressed, results from a mutation in RNA polymerase (159). Should the expression of *pyrA* be modulated in part by the size of the uridine triphosphate and cytidine triphosphate pools through an attenuation type of mechanism analogous to that observed for *pyrBI* in *E. coli*, the uracil sensitivity of the *use* mutant could result from an alteration of a tRNA or a tRNA-modifying enzyme. However, in *E. coli*, where the sequence of the corresponding locus (*carAB*) is known, no attenuation appears to operate.

ARS and AUX *pyrA* mutants of *S. typhimurium* have been studied in some detail. It has been proposed that the formation of a complex between OTCase and CPSase is required for correct subunit assembly of the latter enzyme in the ARS mutants but not in wild-type strains (9). The AUX phenotype is often accompanied by cold sensitivity. Such a phenotype would result from an antagonistic effect of acetylornithine on correct CPSase maturation at nonpermissive temperature, leading to the formation of an enzyme with altered catalytic and regulatory properties (10). This effect is abolished when exogenous arginine curtails acetylornithine synthesis (10).

B. subtilis. Arginine biosynthesis in *Bacillus* spp. follows a linear pathway. There are two CPSases, one regulated by pyrimidines and one regulated by arginine (262, 283). Carbamoylphosphate is not channeled or compartmentalized and mutants in each one of the loci result in arginine or uracil sensitivity (262). Arginine can be used as a carbon or nitrogen source or both. In *B. subtilis* it is degraded by an arginase pathway (82), whereas in *B. licheniformis* arginine is metabolized through either an arginase or an arginine deiminase pathway (45).

The biosynthetic genes of *B. subtilis* are repressed by arginine, while those of the arginase pathway are induced (82; see subsection, "The Arginase Pathway"). Mutants unable to induce the catabolic enzymes were isolated on the basis of their resistance to the arginine analog arginine hydroxamate (29, 135). They map at four unlinked loci: *ahrA* to *C* (233) and *ahrD* (29). The current state of knowledge of metabolic interconnections does not allow us to distinguish between direct and indirect regulatory effects of these mutations. However, *ahrC* mutants also show derepression of the biosynthetic enzymes (135), which suggests the existence of the same type of correlation between synthesis and degradation of arginine as the one observed in yeasts (389).

Seven of the arginine biosynthetic genes are clustered at the *argA-F*/*carAB* locus (we apply here the nomenclature used by Piggot and Hoch [278] where letters follow the order of the biosynthetic steps); the other ones are clustered at the *argGH* locus (136, 203). Recently, the genes at *argA-F*/*carAB* have been ordered by restriction-complementation

mapping after cloning in *E. coli*: *argC-argA-argE-argB-argD-carAB-argF* (235). A promoter site has been found next to *argC* (205), but other promoters may be present within the gene cluster.

The *carB* and *argF* coding regions appear to overlap slightly (S. Baumberg, personal communication). The *argC* promoter region harbors sequences showing homology with *E. coli* arginine operator sites (Baumberg, personal communication). There is no indication, however, that *B. subtilis arg* genes are repressed even partially in *E. coli*. The biosynthetic repressor gene may have been isolated by Margaret C. M. Smith (Baumberg, personal comunication). It complements *ahrC* mutations.

Under aerobic growth conditions or in early stationary phase, the OTCase of *B. subtilis* becomes slightly inducible by arginine (243; Stalon, unpublished data). The physiological relevance of this phenomenon is not evident since *B. subtilis* does not possess a deiminase pathway (243) and since OTCase is not needed for arginine accumulation into spores (244). CPSase is also slightly inducible in stationary cells (263).

Fragmentary nucleotide or amino acid residue sequences are now available for the *argF* (R. Switzer, personal communication; Baumberg, personal communication) and *argC* and *argH* genes (Baumberg, personal communication) of *B. subtilis*. Homologies were found by these authors between *argF* and *argF* and *argI* of *E.coli* (366), *ARG3* of *Saccharomyces cerevisiae* (R. Huygen, unpublished experiments, this laboratory), and human OTCase (143). *argC* shows homology with *argC* of *E. coli* (274) and *argH* shows homology with *ARG4* of *Saccharomyces cerevisiae* (30, 76) and *argH* of *E. coli* (unpublished results, this laboratory).

Transport of Arginine and Related Metabolites

The transport mechanisms responsible for the uptake of arginine and related metabolites carry out an important step in the utilization of these molecules for protein synthesis or as carbon and nitrogen sources. However, as an extensive discussion of the mechanism of arginine transport is outside the scope of this review, the reader is referred to recent papers by Celis (54–56) for what concerns *E. coli*, in which this mechanism has been investigated in detail. From the physiological point of view, a first salient question is whether arginine transport in this organism is regulated and whether it is integrated in the arginine regulon.

Active transport of arginine requires ATP and proceeds through both a high- and a low-affiity system. The latter accounts for most of the arginine uptake at concentrations on the order of 10^{-6} M or higher. It consists of at least two components: an arginine- and ornithine-binding protein (the *abpS* product) and a putative membrane carrier. Arginine and ornithine repress the formation of that system. Regulatory mutants (at the *abpR* gene, closely linked to *abpS* [55]) make high amounts of the *abpS* protein. The dominance pattern of *abpR* mutations is not yet known; however, the data indicate that the *abpR* and *argR* products operate in two independent regulatory circuits (55). It therefore appears unlikely that the low-affinity system is part of the *arg* regulon, but a more extensive dissection of all of the regulatory elements involved in arginine transport is necessary before firm conclusions can be reached.

The high-affinity system is less specific: it also transports ornithine and lysine, and it is repressible by lysine (see reference 54). In *Salmonella* spp., an interaction between the lysine-ornithine-arginine binding protein and the *hisP* product is an essential step in the utilization of arginine as nitrogen source (184).

In *P. putida* (strain ATCC 25592) arginine transport is also mediated by two systems with a high and a low affinity for arginine, respectively (101). In this case, however, it is the former system which displays a narrower substrate specificity (L-arginine specific) than the second one (specific for L-arginine, L-lysine, and L-ornithine). Cultures grown on L-arginine (as C and N source) lack the low-affinity, general basic amino acid permease but are induced for the specific one; the expression of the specific system appears submitted to catabolite repression. In *P. putida* also, a third system transports L-lysine, L-ornithine, and probably D-arginine. Coupled to an arginine racemase, such a system allows utilization of D-arginine as a carbon source by this bacterium. The homologous racemase of *P. graveolens* has been extensively purified and found to be active with lysine, ornithine, and citrulline as well (395). In *P. aeruginosa*, using arginine as energy source in anaerobiosis, an additional arginine-specific uptake system appears to be involved (360).

Little is known of the uptake systems for arginine-derived metabolites (except for polyamines; see reference 343). Among *Pseudomonas* species that efficiently utilize arginine as a carbon source by the arginine succinyltransferase route, only a few are able to grow on succinyl derivatives, and when they do they use only one of these at a reduced rate.

CATABOLISM OF ARGININE

In this section we summarize our present knowledge of the various strategies used by bacteria to degrade arginine. A major aim is to assess the relative importance of these different pathways on the basis of recent information on mutants deficient in catabolic enzymes. Indeed, the variety of mutants now avilable has made it possible to identify alternative pathways, the contribution of which had not been recognized before, and has led to the reinterpretation of the role of some of the several pathways which may occur in the same bacterium. Whenever possible, information regarding control of enzyme synthesis has been included as well. This topic was previously reviewed by Abdelal (4), who also extensively surveyed the properties of many of the enzymes involved in arginine catabolism (Table 3).

Arginase Pathway

The hydrolytic cleavage of arginine to ornithine and urea by arginase is the most thoroughly investigated of the enzymatic mechanisms concerned with arginine degradation in living systems. Arginase has been characterized for various bacterial species, many bacilli (82, 253), the *Agrobacterium-Rhizobium* group (85, 370), cyanobacteria (123, 386), *Proteus* spp. (289), *Streptomyces* spp. (362, 367), *T. aquaticus* (81), and mycobacteria (400).

The fate of ornithine and urea may differ from one organism to another. Nothing is known on the catabolism of ornithine in *Streptomyces* spp., mycobacteria, *T. aquaticus*, and *Proteus* spp. or on urea metabolism in the three former organisms. Urea is generally not utilized by most *Bacillus* strains (253): it is split by urease and used as a nitrogen source by most other organisms. In the cyanobacterium *Synechocystis* sp. strain PCC 6308, ornithine is not metabolized further. It has been suggested that in this bacterium the constitutive arginase-urease system provides only the nitrogen and not the carbon to the cell (386).

TABLE 3. Enzymes of arginine catabolism

Name	EC no.	Reactions catalyzed
Arginase	3.5.3.1	Arginine = ornithine + urea
Ornithine transaminase	2.6.1.13	Ornithine + 2-oxoglutarate = glutamate semialdehyde + glutamate
Pyrroline-5-carboxylate dehydrogenase	1.5.1.12	1-Pyrroline-5-carboxylate + NAD^+ + H_2O = glutamate + NADH
ADI	3.5.3.6	Arginine + H_2O = citrulline + NH_3
OTCase	2.1.3.3	P_i + citrulline = carbamoylphosphate + ornithine
Carbamate kinase	2.7.2.2	Carbamoylphosphate + ADP = ATP + NH_3 + CO_2
Arginine decarboxylase	4.1.1.19	L-Arginine = agmatine + CO_2
Agmatine ureohydrolase	3.5.3.11	Agmatine + H_2O = putrescine + urea
Ornithine decarboxylase	4.1.1.17	L-Ornithine = putrescine + CO_2
Agmatine deiminase	3.5.3.12	Agmatine + H_2O = *N*-carbamoylputrescine + NH_3
N-Carbamoylputrescine hydrolase	3.5.3.–	*N*-Carbamoylputrescine + H_2O = putrescine + CO_2 + NH_3
Putrescine carbamoyltransferase	2.1.3.6	Carbamoylphosphate + putrescine = carbamoylputrescine + P_i
Putrescine transaminase	2.6.1.–	Putrescine + 2-oxoglutarate = L-glutamate + 4-aminobutyraldehyde
Putrescine oxidase	1.4.3.10	Putrescine + O_2 = 4-aminobutyraldehyde + NH_3
4-Aminobutyraldehyde dehydrogenase	1.2.1.19	4-Aminobutyraldehyde + NAD = 4-aminobutyrate + NADH + H^+
4-Aminobutyrate transaminase	2.6.1.19	4-Aminobutyrate + 2-oxoglutarate = L-glutamate + succinate semialdehyde
Succinate semialdehyde dehydrogenase	1.2.1.16	Succinate semialdehyde + NADP = succinate + NADPH + H^+
	1.2.1.24	Succinate semialdehyde + NAD = succinate + NADH + H^+
Arginine succinyltransferase	2.3.1.–	L-Arginine + succinyl CoA = N^2-succinylarginine + CoA
Succinylarginine dihydrolase	(3.5.3.–)	N^2-succinylarginine-N^2-Succinylornithine + $2NH_3$ + CO_2
Succinylornithine aminotransferase	2.6.1.–	N^2-Succinylornithine + 2-oxoglutarate = N^2-succinylglutamate semialdehyde + L-glutamate
Succinylglutamate semialdehyde dehydrogenase	1.2.1.–	N^2-Succinylglutamate semialdehyde + NAD = N^2-succinylglutamate + NADH + H^+
Succinylglutamate desuccinylase	3.5.1.–	N^2-Succinylglutamate = succinate + glutamate
Arginine aminotransferase	2.6.7.–	L-Arginine + 2-oxoglutarate = 2-ketoarginine + L-glutamate
Arginine 2-monooxygenase	1.13.12.1	L-Arginine + O_2 = 4-guanidinobutyramide + CO_2 + H_2O
4-Guanidinobutyramidase	5.1.5.–	4-Guanidinobutyramide + H_2O = 4-guanidinobutyrate + NH_3
Arginine racemase	5.1.1.9	L-Arginine-D-arginine
Arginine oxidase	1.4.3-	Arginine + H_2O + O_2-ketoarginine + NH_3 + H_2O_2
2-Ketoarginine decarboxylase	4.1.1-	2-Ketoarginine-guanidinobutyraldehyde + NH_3
4-Guanidinobutyraldehyde oxidoreductase	1.2.1-	4-Guanidinobutyraldehyde + NAD-4-guanidinobutyrate + NADH + H^+
Guanidinobutyrase	3.5.3.7	4-Guanidinobutyrate + H_2O = 4-aminobutyrate + urea
Urease	3.5.3.1	Urea = $2NH_3$ + CO_2
Arginine racemase	5.1.1.9	L-Arginine = D-arginine
Ornithine racemase	5.1.1.12	L-Ornithine = D-ornithine
D-Ornithine 4,5-aminomutase	5.4.3.5	D-Ornithine = D-*threo*-2,4-diaminopentanoate
2-4-Diaminopentanoate dehydrogenase	1.4.1.12	2,4-Diaminopentanoate + H_2O + NAD(P^+) = 2-amino-4-oxopentanoate + NH_3 + NAD(P)H
2-Amino-4-pentanoate CoA transferase	2.8.3.–	2-Amino-4-pentanoate + CoA = L-alanine + acetyl CoA
Ornithine cyclase	4.3.1.12	L-Ornithine = L-proline + NH_3
Proline racemase	5.1.1.4	L-Proline = D-proline
Proline dehydrogenase	1.5.99.8	Proline + acceptor + H_2O = pyrroline-5-carboxylate + reduced acceptor
D-Proline reductase	1.4.4.1	D-Proline + dihydrolipoate = 5-aminopentanoate + lipoate

Utilization of ornithine as carbon and nitrogen sources by *Bacillus* strains involves a transamination with 2-ketoglutarate to form glutamate and glutamate semialdehyde, the latter being itself converted to pyrroline-5-carboxylate by chemical cyclization (Fig. 4). The last reaction in this pathway, the oxidation of pyrroline-5-carboxylate to glutamic acid, is shared with the proline catabolic pathway (82, 293). Two different catabolic pyrroline-5-carboxylate dehydrogenases belong to either arginine or proline catabolism on the basis of their regulation have been detected in the *Bacillus* sp. used by De Hauwer et al. (82); the strain is formally identified as *B. licheniformis* rather than *B. subtilis*. The results of Laishley and Bernlohr (187) with *B. licheniformis* A-5 and those of Baumberg and Harwood (28) with *B. subtilis* also suggest the absence of any other activity common to both arginine and proline catabolic pathways than pyrroline 5-carboxylate dehydrogenase. In these strains the enzymes of the arginase pathway are induced by arginine, citrulline, and ornithine and also by proline. The significance of proline induction is not clear. It was suggested that pyrroline-5-carboxylate or glutamate semialdehyde, at which the arginine and proline catabolic pathways converge, is the true inducer (187).

In *B. licheniformis* the arginase pathway enzymes are subject to strong catabolite repression during growth on glucose (45, 187). The intensity of repression seems to be much weaker in *B. subtilis* (28). In several *Bacillus* species, the arginase pathway is also subject to nitrogen catabolite repression. In *B. licheniformis*, glutamine antagonizes the induction of the arginase pathway. This effect occurs only in media containing a good carbon source. Neither ammonia nor glutamate entails a significant reduction of enzyme levels. In this organism glutamine is a better nitrogen source than arginine, glutamate, or ammonia (45). In the *B. subtilis* strain used by Baumberg and Harwood (28) the arginase pathway is also repressed by glutamine and, in addition,

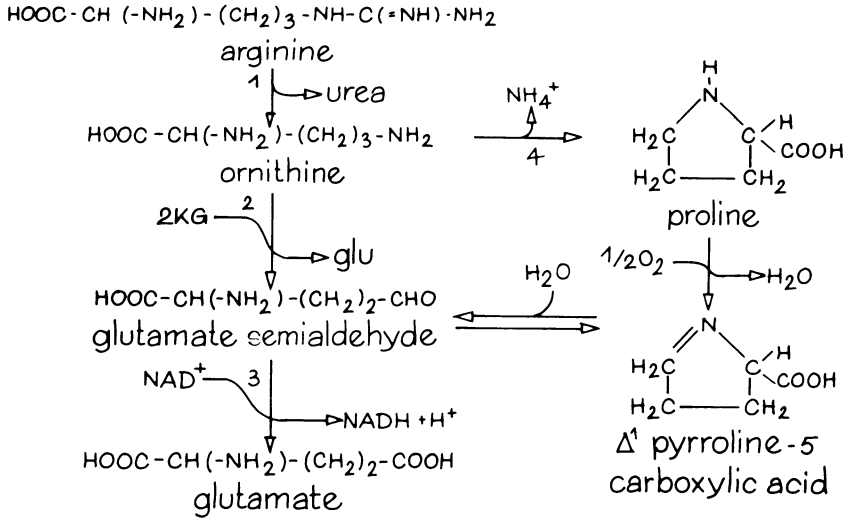

FIG. 4. Arginase pathway. Enzymatic steps are as follows: (1) arginase, (2) ornithine transaminase, (3) pyrroline-5-carboxylate dehydrogenase, (4) ornithine cyclase.

ammonia. However, in other *Bacillus* species nitrogen catabolic repression does not appear to be involved in the regulation of arginase synthesis (317).

The arginine metabolic system of *B. subtilis* has been the subject of some physiological and genetic studies. The biosynthetic enzymes are repressed by arginine (28). No repression is observed in the presence of citrulline or ornithine, which are able to induce the arginase pathway. Mutants resistant to the arginine analog arginine hydroxamate (*ahrC*) present alterations in the expression of the arginase pathway genes and a simultaneous loss of repressibility of the arginine biosynthetic genes (29, 135) (see "Control of Enzyme Synthesis in Other Procaryotes").

In the genus *Agrobacterium*, arginine may be used as a nitrogen source by an inducible arginase and a constitutive urease. Utilization of ornithine as nitrogen source results from the action of a constitutive ornithine-5-transaminase. Many *Agrobacterium* strains are also able to use arginine or ornithine as a carbon source. However, some *Agrobacterium* strains unable to grow on arginine or ornithine as a carbon source display this ability when they harbor a wild-type octopine or nopaline Ti plasmid (96). The utilization of ornithine as a carbon source proceeds thus via cyclization of ornithine into proline, proline being then converted into glutamic acid, most likely by a proline oxidase and a pyrroline-5-carboxylate dehydrogenase as indicated by the fact that mutants lacking proline oxidase become unable to grow on arginine or ornithine as a carbon source (85). Schardl and Kado (311) claimed that an ornithine dehydrogenase responsible for ornithine utilization was both pTi and chromosome encoded in a C58 strain of *A. tumefaciens*. Dessaux and co-workers, however, were unable to detect any dehydrogenase activity in all of the strains they tested, including C58 (85).

ADI Pathway

Arginine deiminase (ADI), OTCase, and carbamate kinase constitute the deiminase pathway which catalyzes the conversion of arginine into ornithine, ammonia, and carbon

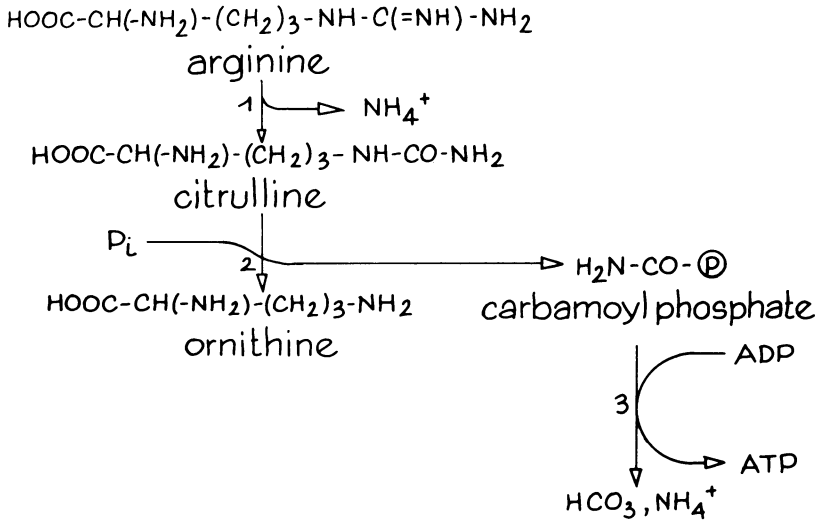

FIG. 5. ADI pathway. Enzymatic steps are as follows: (1) ADI, (2) OTCase, (3) carbamate kinase.

dioxide with the formation of 1 mol of ATP per mol of arginine consumed (Fig. 5).

The pathway is widely distributed among procaryotic organisms. It has been detected in lactic bacteria (83), bacilli (45, 253), *Pseudomonas* spp. (337, 338), *Aeromonas* spp. (340), clostridia (227, 316, 368), *Mycoplasma* spp. (25, 314, 326), *Spiroplasma* spp. (147), *Spirochaeta* spp. (38), halobacteria (92), and cyanobacteria (386).

Arginine serves as sole energy source for *Streptococcus faecalis* (83, 325), *Mycoplasma* spp. (315), *Bacillus* spp. (45), *P. aeruginosa* (360), halobacteria (134), and *Spirochaeta* spp. (38). Arginine utilization by the ADI pathway in most of these strains is characterized by an abundant ornithine excretion, indicating that only the guanidino group of arginine is used. However, *Treponema denticola* and some clostridia are able to dissimilate ornithine by either the action of an ornithine cyclase yielding proline (65, 331) or by a Stickland reaction producing 5-aminovalerate (93). Ornithine is also used for putrescine synthesis.

The data concerning *Mycoplasma* spp. give a confusing picture. Schimke and associates (314, 315) reported that arginine constitutes a major source of energy and that arginine is rapidly converted into excreted ornithine by the ADI pathway. The latter observation contrasts results of Fenske and Kenny (103) who, using the same strain as Schimke (*Mycoplasma hominis* 07) (formally classified as *M. arthriditis*), showed that these cells do not excrete ornithine but rather citrulline. The use of different media in which different nutrients are limiting is probably the cause of these conflicting observations. In the semisynthetic medium used by Fenske and Kenny, repression of OTCase or carbamate kinase by an excess of energy sources other than arginine may result in excretion of citrulline.

Data concerning arginine utilization by *Streptococcus faecium* also lead to some difficulties of interpretation. In a synthetic medium the simultaneous presence of glucose and arginine are required for the exponential growth of *Streptococcus faecalis* strains now classified as *Streptococcus faecium* (264). This phenomenon was explained by the presence in cell extracts of *Streptococcus faecium* of a carbamoylphosphate-glucose phosphotransferase activity catalyzing the transfer of the phosphate group of carbamoylphosphate to glucose, yielding glucose-6-phosphate and thereby initiating glycolysis (264). In addition, carbamoylphosphate was found to contribute to glucose uptake by the cellular phosphotransferase system. ATP and adenosine disphosphate (ADP), which act as inhibitors of glycolysis, were found to inhibit arginine deiminase and AMP was found to stimulate it (V. W. Pendey, personal communication). No carbamate kinase could be detected under those circumstances; utilization of arginine therefore appeared to be strictly linked to glycolysis. However, this coherent body of evidence is at first sight difficult to reconcile with the observation by Deibel (83) that the ADI pathway is induced only after exhaustion of the glucose and with the finding of a carbamate kinase in the presence of glucose and arginine by Thorne and Jones (346). Differences in medium composition and growth conditions are probably responsible for these discrepancies.

A similar situation occurs in *Streptococcus mitis* (169). Intracellular accumulation of free glucose is driven by the proton motive force when glucose uptake by the phosphotransferase system is low. Free glucose, in contrast to glucose-6-phosphate accumulated via the phosphotransferase system, is metabolized either via glycolysis or via glucose-1-phosphate towards glycogen; ATP is less effective than carbamoylphosphate for the generation of glucose-1-phosphate. Since the phosphotransferase system is repressed or inactivated by low-pH conditions which promote proton gradient-driven sugar uptake, it would appear that media and culture conditions may be important factors in regulating the carbamoylphosphate flow towards its various phosphoryl acceptors, ADP, or glucose.

Besides its energy-generating function, the ADI pathway may provide nitrogen when arginine is the only available source for organisms such as in *Aeromonas formicans* (340) and *B. licheniformis* (45). In aerobiosis, arginine utilization by the former organism passes through succinylarginine formation, while for the latter it proceeds via arginase. In anaerobiosis and in both organisms, the aerobic pathway disappears and consumption of arginine results from the appearance of the ADI pathway. Anaerobic growth of *B. licheniformis* is observed on mineral medium supplemented with glucose plus ammonia, glucose plus arginine, or pyruvate plus arginine but not on pyruvate plus ammonia. These observations indicate that, besides its nitrogen-providing function, arginine degradation by the ADI pathway also provides energy in the absence of another good energy source (45). Nitrogen catabolite repression of the ADI pathway by ammonia occurs in the presence of a good energy source such as glucose.

Control of energy provision. Control of energy provision via the ADI pathway was mostly investigated for *Streptococcus faecalis* and various lactic bacteria, as well as for *P. aeruginosa*, *B. licheniformis*, *Aeromonas formicans*, and *Halobacterium salinarium*. Mutants affected in either structural or regulatory genes provided some insight into the functioning of the pathway.

Energy depletion is an essential signal for inducing the ADI pathway enzymes in most of the bacteria so far studied: energy depletion alone is enough to induce the pathway in *Pseudomonas* spp. (294, 337); arginine, however, enhances twofold the inductive response of enzyme synthesis produced under these conditions in *P. aeruginosa* PAO (124, 217); both energy depletion and arginine are required for the induction of the pathway in *Streptococcus faecalis* ATCC 11700 (135), *B. licheniformis* ATCC 14580 (45), and *Aeromonas formicans* NCIB 9232 (340). Repression of the pathway occurs under conditions which promote the energy status of the cells: presence of a fermentable substrate or other energy sources or both as well as aerobic and anaerobic respiration (for the denitrifying *B. licheniformis* and *P. aeruginosa*). Exogenous cAMP, a signal of energy deficit, antagonizes glucose or oxygen repression of the ADI pathway of *Aeromonas* spp. (340). Exogenous ATP and uridine triphosphate substantially repress enzyme formation in *P. aeruginosa* (217). In the aerotolerant *Streptococcus faecalis*, oxygen in the presence of hematin was found to increase glucose repression of enzyme synthesis in the ADI pathway (325). Hematin enables *Streptococcus faecalis* to synthesize porphyrin and thus to develop a rudimentary electron transport system and to become capable of oxidative phosphorylation. All these observations suggest that ATP itself or one of its derivatives (for instance, cAMP in *Aeromonas* spp.) may control the ADI pathway. In the organisms mentioned above, synthesis of the ADI enzymes appears coordinated (217, 325; Stalon, unpublished observations).

Besides this common response to energy depletion, procaryotes exhibit a variety of regulatory patterns of the ADI pathway, as revealed by the isolation of mutants or natural strains unable to use arginine as an energy source. In *Streptococcus faecalis* such mutants are affected in at least

one of the three enzymes of the pathway or in the uptake system, or display a low level of all three enzymes, suggesting the existence of a common element for their induction. In *Streptococcus sanguis*, which seems to follow the same regulatory pattern as *Streptococcus faecalis*, a barotolerant mutant has been isolated which escapes glucose repression of all three enzymes (104). In *Streptococcus lactis* isolated from a dairy, the synthesis of the first two enzymes of the pathway seems to be glucose controlled, the carbamate kinase activity being constitutive (72). Some *Streptococcus lactis* strains unable to use arginine are blocked in both ADI and OTCase. This observation is reminiscent of the natural dairy strains of *Streptococcus cremoris* that have constitutive carbamate kinase but lack ADI or ADI and OTCase. In those strains, restoration of both ADI and OTCase to the level normally found in *Streptococcus lactis* may be obtained by a single point mutation (72). As the two species are closely related, it is conceivable that *Streptococcus cremoris* is a variety of *Streptococcus lactis* which has lost the first two enzymes of the ADI pathway or the possibility to induce them.

Mutants with mutations affecting the ADI route were first obtained with *P. aeruginosa* as follows: (i) anabolic OTCase-deficient mutants were isolated (125); (ii) revertants were selected in which the molecular and regulatory properties of catabolic OTCase were altered in such a way (see "Evolutionary Considerations") that the enzyme could fulfill an anabolic function (124); (iii) citrulline auxotrophs were selected in which no OTCase activity could be detected (124) (such strains, when induced for the ADI pathway, produced citrulline from arginine); (iv) citrulline-nonproducing mutants were selected which lacked ADI activity (218).

Of aerobic *Pseudomonas* spp., Haas and associates (360) observed that *P. aeruginosa* can grow anaerobically with arginine as energy source. This property was used to select strains affected in the ADI route, using as phenotype the fact that arginine could not sustain the anaerobic growth of these mutants any more. As for *Streptococcus faecalis*, these mutants were found to be affected either in the anaerobic arginine uptake system or in at least one enzyme of the pathway or to be mutated in a control element required for pleiotropic enzyme induction. The mutations *arcA* (ADI), *arcB* (OTCase), *arcC* (carbamate kinase), and *arcD* (anaerobic uptake system) are all highly cotransducible in the 17-min chromosome region (360). Interestingly, *P. aeruginosa* is the only fluorescent *Pseudomonas* sp. able to grow fermentatively in the presence of arginine. *P. putida*, *P. fluorescens*, and *P. mendocina* are unable to do so (360).

For *B. licheniformis*, mutants deficient in carbamate kinase were found among strains unable to use arginine as energy source in anaerobiosis (K. Broman, Ph.D. thesis, University of Brussels, Brussels, Belgium, 1978). They failed to grow on pyruvate plus arginine in anaerobiosis but still retained the ability to do so in aerobiosis due to the presence of the arginase route (Broman, Ph.D. thesis). The deiminase pathway can fulfill the nitrogen requirement. Regulatory mutants with mutations affecting the ADI pathway were selected from a strain unable to induce both arginase and ornithine transaminase for their ability to use arginine in aerobiosis as the sole nitrogen source in the presence of glucose (45). In these mutants, induction of the pathway by arginine is no longer repressed by nitrogen. Oxygen or nitrate are without effect and glucose repression is largely reduced, thus indicating that one element is shared by the mechanisms respectively responsible for nitrogen, glucose, O_2, and NO_3^- repression. Secondary mutants of *B. licheniformis* isolated for their ability to utilize arginine as a carbon source in aerobiosis either are revertants having regained the ability to induce the synthesis of arginase and ornithine transaminase or are inducible for ornithine transaminase only, no arginase being synthesized; in these organisms the ornithine formed by the ADI route is used via the transaminase (Stalon, unpublished experiments).

Halobacteria are able to grow anaerobically with the ATP derived from the light energy harvested by the bacteriorhodopsin proton excretion system. This system is synthesized only under the condition of limited oxygen supply but not in darkness or in the total absence of oxygen. Under these conditions, arginine can replace oxygen or light as energy sources by the ADI route. Mutants unable to use arginine as the energy source to grow in anaerobiosis have been selected, but their lesions have not yet been characterized (134).

The expression of the ADI pathway in clostridia is correlated with sporulation (227, 369). While OTCase and carbamate kinase in *Clostridium sporogenes* appear constitutive, ADI seems to be regulated at the levels of both gene expression and enzyme activity. Carbon sources known to repress sporulation also repress ADI synthesis (369).

The catabolic OTCase of the fluorescent *Pseudomonas* group as well as of *P. mendocina* is also subject to enzymotropic regulation (Stalon, unpublished results). Cooperative binding of phosphate, citrulline, and carbamoylphosphate has been observed. In addition, the OTCases of *P. putida* and *P. aeruginosa*, the allosteric properties of which have been thoroughly studied, are regulated by a variety of metabolic signals activating or inhibiting its activity (334). Pyrimidine and purine triphosphonucleotides, as well as the polyamines putrescine, spermine, and spermidine, inhibit the activity by increasing the threshold substrate concentration required to activate the enzyme, whereas the nucleotide monophosphate restores the normal kinetic behavior. Since the catabolic OTCase is integrated in an energy-producing pathway, the significance of the activation by AMP, a signal of energy deficit, and the inhibition by ATP, an index of the high energy status of the cell, are easily understood. Since the putrescine content is high in proliferating cells and low in starving cells, the inhibitors putrescine, spermidine, and spermine should be envisioned as signals reflecting the availability of good carbon and nitrogen sources rather than as end products of the ADI route.

Arginine Succinyltransferase Pathway

The recently discovered arginine succinyltransferase pathway (335, 361) accounts for the dissimilation of the carbon skeleton of arginine in *Aeromonas formicans*, in *K. aerogenes*, and in most of the *Pseudomonas* strains of group I (*P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. mendocina*, and *P. aureofaciens*), group II (*P. cepacia* and *P. caryophylii*), and group V (*P. indigofera* and *P. doudoroffii*) as defined by Palleroni et al. (256). Most of these organisms have, however, other pathways for arginine dissimilation whose function is to provide either polyamines when arginine is abundant (see the arginine decarboxylase pathway) or energy by the ADI route (see preceding section). However, in *P. cepacia*, the arginine succinyltransferase route is the sole arginine catabolic pathway. This route follows the five reactions shown in Fig. 6. The first reaction in this sequence is catalyzed by arginine succinyltransferase; N^2-succinylarginine dihydrolase then converts N^2-succinylarginine into N^2-succinylornithine, ammonia, and carbon dioxide. The

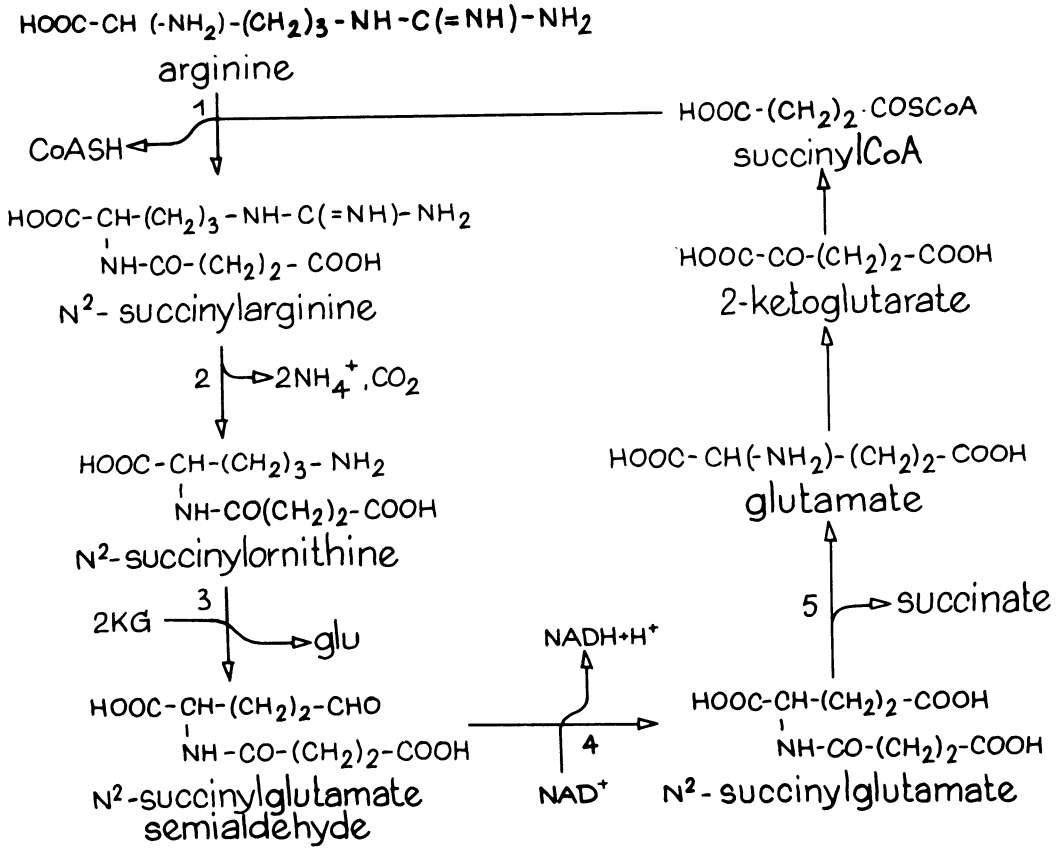

FIG. 6. Arginine succinyltransferase pathway. Enzymatic steps are as follows: (1) arginine succinyltransferase, (2) succinylarginine dihydrolase, (3) succinylornithine aminotransferase, (4) succinylglutamate semialdehyde dehydrogenase, (5) succinylglutamate desuccinylase.

third step is catalyzed by N^2-succinylornithine aminotransferase, which transfers the delta nitrogen atom of N^2-succinylornithine to 2-ketoglutarate, giving glutamate and N^2-succinylglutamate semialdehyde. The latter is oxidized by a dehydrogenase into N^2-succinylglutamate, which is cleaved into succinate and glutamate. The presence of the arginine succinyltransferase route in *K. aerogenes* MK53 brings into question the existence of the arginine transamidinase activity suggested by Friedrich and co-workers (107, 108) to explain arginine catabolism in this strain. The postulated existence of this activity was based on the observations that (i) arginine donates its guanidino group to hydroxylamine and forms ornithine (108) and (ii) cells grown with arginine synthesize an ornithine transaminase activity, this enzyme having, however, more affinity for acetylornithine than for ornithine (107). In our hands, the arginine-inducible acetylornithine aminotransferase activity of *Klebsiella* spp. may also transaminate succinylornithine, the K_m for succinylornithine being four times lower than that observed for acetylornithine (335).

As expected from their physiological role, the syntheses of these enzymes in *P. cepacia*, *P. aeruginosa*, and *P. putida* are subject not only to induction by arginine, but also to carbon catabolite repression by succinate and nitrogen catabolite repression by glutamine. Ammonia has little effect on the synthesis of these enzymes.

P. aeruginosa mutants which cannot grow aerobically on arginine have been isolated (292, 371); they are blocked in structural genes coding for at leat one activity of the pathway or fail to induce synthesis of all of the enzymes (D. Haas, C. Vander Wauven, and V. Stalon, manuscript in preparation). All of these mutations are clustered in the 90-min region of the *P. aeruginosa* chromosome and are unlinked to the other loci involved in arginine catabolism (Haas, personal communication).

For *P. putida*, a mutant impaired in the succinylarginine dihydrolase activity can still grow on arginine but at a lower rate than the wild-type strain. The growth yield is also largely reduced because 50% of the arginine consumed is recovered in the culture fluid as N^2-succinylarginine. The ability of a *P. putida* strain to grow on arginine in spite of a defect in the arginine succinyltransferase route is due to the presence of another arginine catabolic pathway, the arginine oxidase route (see next section).

Arginine Transaminase, Oxidase, and Oxygenase Pathways

The coryneform bacterium *Arthrobacter simplex* can catalyze the transamination between arginine and 2-ketoglutarate to produce 2-ketoarginine and glutamate (347). The arginine transaminase was extensively purified and was found to transaminate arginine (100%) but also citrulline (16%) and alanine (9.5%) (344). In this organism, 2-ketoarginine appears to undergo hydrolytic cleavage of the guanidino group to yield urea and 2-ketoornithine, the fate of which remains unknown. This observation is at first sight difficult to reconcile with the fact that arginine transaminase activity is not induced only by arginine and 2-ketoarginine

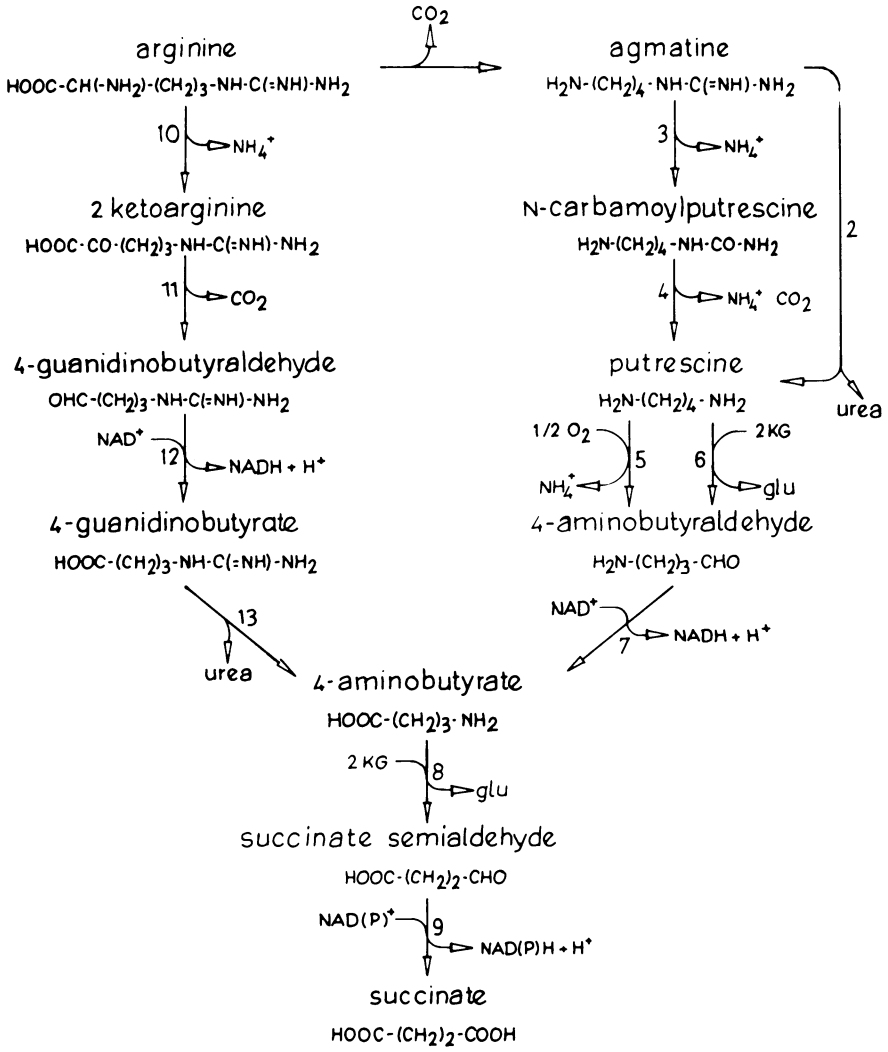

FIG. 7. Arginine decarboxylase and arginine oxidase pathways. Enzymatic steps are as follows: (1) arginine decarboxylase, (2) agmatine ureohydrolase, (3) agmatine deiminase, (4) *N*-carbamoylputrescine hydrolase, (5) putrescine oxidase, (6) putrescine transaminase, (7) 4-aminobutyraldehyde dehydrogenase, (8) 4-aminobutyrate transaminase, (9) succinate semialdehyde dehydrogenase, (10) arginine oxidase, (11) 2-ketoarginine decarboxylase, (12) 4-guanidinobutyraldehyde oxidoreductase, (13) 4-guanidinobutyrase.

but also by guanidinobutyrate. Induction by the latter compound might of course be gratuitous, due to its structural similarity to the true inducer.

2-Ketoarginine may also be produced by an arginine oxidase such as in the cyanobacterium *Synecoccocus* sp. strain PCC 6308 (or *Anacystis nidulans*), in which this enzyme, a flavoprotein, is responsible for the oxidation of arginine in the dark (282). The native enzyme has an MW of 98,000 and is composed of two identical subunits. L-Arginine oxidase also catalyzes the oxidation of lysine, ornithine, and histidine. It is inhibited by divalent cations whose inhibitory effect can be reversed by ATP and ADP (281). The L-arginine oxidase of cyanobacteria is part of photosystem II and may thus play a dual role, acting as either an electron-transferring protein in photosynthesis in the presence of certain metal ions or an L-amino acid oxidase in the absence of them (282). The fate of 2-ketoarginine in *Synecoccocus nidulans* is not known. In other organisms such as *P. putida*, in which an arginine oxidase seems also to be involved in 2-ketoarginine formation, this substrate may be used as sole carbon and nitrogen source (226). The pathway is described in Fig. 7. A 2-ketoarginine decarboxylase yields guanidinobutyraldehyde, which is oxidized into guanidinobutyrate by the action of a dehydrogenase (359).

Actinomycetes and some corynebacteria may also produce guanidinobutyrate from arginine by a pathway which resembles that of *P. putida*. *Streptomyces griseus* degrades arginine via 4-guanidinobutyramide, which is then oxidized into 4-guanidinobutyrate (363–365). The first step is catalyzed by an arginine decarboxioxidase and the second is catalyzed by a guanidinobutyramidase. The enzymes are induced by growing the cells on arginine as sole carbon and nitrogen source (364). Induction of guanidinobutyramidase by arginine in *Brevibacterium helvolum* (ATCC 11822) suggests that this organism degrades arginine by the same pathway as *Streptomyces griseus* and the other actinomycetes (T. Yorifugi, personal communication).

The pathway of guanidinobutyrate utilization is similar in the various organisms examined so far. Guanidinobutyrase cleaves guanidinobutyrate into urea and 4-aminobutyrate, which in the presence of 2-ketoglutarate produces glutamate and succinate semialdehyde through the action of a

transaminase. Succinate semialdehyde is then oxidized into succinate by a dehydrogenase (see below).

In *P. putida* studies on the regulation of the oxidase pathway suggest the existence of at least five independent regulatory entities: (i) the L-arginine-specific transport system, which is induced by arginine (101); (ii) arginine oxidase; (iii) 2-ketoarginine decarboxylase; (iv) guanidinobutyraldehyde dehydrogenase and guanidinobutyrase (359). (v) The subsequent enzymes of the pathway are induced by 4-aminobutyrate or its precursors guanidinobutyrate, agmatine, and putrescine and also by arginine (Legrain and Stalon, manuscript in preparation) (see next section); succinic semialdehyde dehydrogenase is also controlled by succinic semialdehyde precursors such as parahydroxyphenylacetate (24) or by hydroxybutyrate (246).

When 2-ketoarginine and guanidinobutyrate are utilized by *P. putida* as the sole carbon and nitrogen source, guanidinobutyrase induction leads to an abundant release of urea into the medium, 1 mol of urea being produced per mol of substrate. When arginine is the sole carbon and nitrogen source, induction of guanidinobutyrase is only 40 to 60% of that observed with the substrate of the enzyme and about 50% of the arginine is degraded to release urea in the culture medium, the remainder of arginine being used via the arginine succinyltransferase route (Legrain and Stalon, in preparation).

Mutants of *P. putida* impaired in guanidinobutyrase activity are unable to use 2-ketoarginine or guanidinobutyrrate; they are still able to grow on arginine as sole carbon and nitrogen source, via the arginine succinyltransferase pathway, but with reduced rate and yield. 2-Ketoarginine and guanidinobutyrate excretion is observed under these conditions (Legrain and Stalon, in preparation).

Some induction of guanidinobutyrase in arginine-grown cells was also observed with *P. aureofaciens*, *P. chlororaphis*, and one *P. fluorescens* strain (394). Although *P. fluorescens* ATCC 13525 and *P. aeruginosa* use 2-ketoarginine and guanidinobutyrate well, no induction of guanidinobutyrase or urea excretion can be observed when these strains are grown on arginine; this suggests that they do not possess the L-arginine oxidase and rather utilize arginine via the succinyltransferase pathway. In line with this observation, mutants of *P. aeruginosa* blocked in guanidinobutyrase retain the ability to utilize arginine as the wild-type strain does (Haas, personal communication).

Arginine Decarboxylase Pathway

Extensive work with both eucaryotes and procaryotes has shown that putrescine, or its derivatives, is required for optimal growth (see references 342 and 343). In addition, putrescine can be utilized by bacteria as both carbon and nitrogen source. Putrescine is formed by the decarboxylation of either ornithine or arginine into agmatine, which is then converted into putrescine either directly or indirectly via carbamoylputrescine. Which route is taken depends on the regulatory status of the arginine pathway, since arginine blocks ornithine formation via feedback inhibition, and on the type of arginine catabolism used (whether it produces ornithine or not). Thus arginine prototrophic organisms growing on minimal medium and organisms that utilize arginine via arginase synthesize putrescine from ornithine. In arginine-supplemented medium and when arginine catabolism does not produce ornithine, putrescine is derived by decarboxylation of arginine rather than of ornithine.

Ornithine decarboxylase activity is present in enterobacteria (228), *Pseudomonas* spp. (216), *Agrobacterium* spp. (330), acetobacteria (260), mycobacteria (21, 200), *Clostridium* spp. (259), *Lactobacillus* spp. (122), and methanobacteria (313). Arginine decarboxylase is found in enterobacteria (228), *Aeromonas* and *Pseudomonas* spp. (216, 337), and mycobacteria (400).

Further metabolism of agmatine and putrescine depends on the organism considered. Agmatine may be cleaved into putrescine and urea by an agmatinase as in enterobacteria and mycobacteria or hydrolyzed by an agmatine deiminases into carbamoylputrescine and ammonia as in *Pseudomonas* spp., *Aeromonas* spp., and lactic bacteria. Putrescine itself (as obtained directly via agmatinase or from carbamoylputrescine) may either accumulate in the medium (as in lactic bacteria) or be utilized further as a carbon or nitrogen source or both. The first step of putrescine metabolism requires either oxidation of one nitrogen atom of putrescine to give ammonia and 4-aminobutyraldehyde or transamination between putrescine and 2-ketoglutarate to give glutamate and 4-aminobutyraldehyde. The latter compound is then oxidized into 4-aminobutyrate by a dehydrogenase. The subsequent steps of 4-aminobutyrate utilization were described previously for the arginine oxidase route: a transaminase and a dehydrogenase produce succinate via the formation of succinate semialdehyde.

These various alternatives are depicted in Fig. 7. Details are given in the subsections below.

Lactic bacteria. In *Streptococcus faecalis* ATCC 11700 and some lactic bacteria devoid of arginine decarboxylase, agmatine may be used as an energy source by the consecutive action of a three-enzyme system analogous to the ADI route occurring in the same organism (324, 325).

Agmatine deiminase, putrescine carbamoyltransferase, and carbamate kinase constitute the agmatine deiminase route, which catalyzes the conversion of agmatine into putrescine, ammonia, and carbon dioxide with the concomitant formation of 1 mol of ATP per mol of agmatine consumed. Putrescine carbamoyltransferase from *Streptococcus faecalis* ATCC 11700 has been purified to homogeneity; as the anabolic OTCase, it is a trimer of identical subunits of MW 38,000. In addition, this enzyme carbamoylates ornithine at, however, one-tenth of the rate observed with the substrate putrescine, whereas the K_m for ornithine is 1.3 mM against 0.003 mM for putrescine (384).

E. coli. Two types of ornithine and arginine decarboxylases occur in some *E. coli* strains, the catabolic and the biosynthetic enzymes. Catabolic ornithine and arginine decarboxylases are induced under special growth conditions such as rich media, semianaerobiosis, low pH, and the presence of their respective substrates. These enzymes probably constitute a defense mechanism against acidity (Becker, Fed. Proc. **26**:812, 1967).

The properties of *E. coli* arginine decarboxylase and ornithine decarboxylase have been reviewed by Tabor and Tabor (342, 343; Glansdorff, in press). They will not be discussed here.

Wild-type *E. coli* cells do not use arginine or its derivatives as a carbon source but use them well as a nitrogen source. Agmatinase cleaves agmatine into urea and putrescine. No urease being present in *E. coli*, urea accumulates in the medium (231). Putrescine and 2-ketoglutarate transaminate to give 4-aminobutyraldehyde and glutamate. The former compound is then oxidized into 4-aminobutyrate; the subsequent intermediates are succinic semialdehyde and succinate. The entire pathway (Fig. 7) from arginine onwards is probably present in *E. coli* B (180, 181), which can use arginine, putrescine, or 4-aminobutyrate

but not in some *E. coli* K-12 strains which do not use 4-aminobutyrate due to a permeability barrier and the low level of enzymes degrading this compound (88, 89). Similarly, Satishchandran and Boyle (310) found that none of their *E. coli* K-12 strains was able to utilize agmatine as a nitrogen source, although Shaibe et al. (322) as well as ourselves observed that agmatine may be used as a nitrogen source with strains of our collection (339). As expected, a mutant blocked in arginine decarboxylase does not use arginine, cells lacking agmatinase are impaired in both arginine and agmatine utilization, and cells with no ornithine decarboxylase cannot utilize ornithine (321).

As *E. coli* K-12 produces no urease, putrescine transaminase is responsible for the entry of ammonia in the metabolism. Kim (181) described mutants of *E. coli* B which are capable of utilizing putrescine as sole carbon and nitrogen source, due to an increase in activity of the transaminase. Mutants of *E. coli* K-12 blocked in putrescine transaminase do not grow on ornithine, arginine, agmatine, or putrescine as nitrogen source (311). Although *E. coli* K-12 cannot normally grow on 4-aminobutyrate as sole nitrogen source (see above), mutants acquire this ability by a mutation in the *gabC* gene, a regulatory gene which controls the synthesis of the structural gene coding for 4-aminobutyrate transaminase and succinic semialdehyde dehydrogenase (88, 89). Secondary mutants derived from the 4-aminobutyrate-utilizing strains were able to use it as sole carbon source due to enhanced permeability of the cells to this compound (88) and possibly also to the gratuitous induction by succinate semialdehyde of a second succinate semialdehyde dehydrogenase which acts preferentially with nicotinamide adenine dinucleotide (NAD) in addition to the 4-aminobutyrate NAD phosphate (NADP)-inducible succinate semialdehyde dehydrogenase encoded by the *gab* locus (86, 222, 223).

E. coli B also synthesizes two distinct succinic semialdehyde dehydrogenases when grown on 4-aminobutyrate (86). The NADP-dependent enzyme is highly correlated with 4-aminobutyrate transaminase, while the NAD-specific counterpart is induced by *para*-hydroxyphenylacetate or succinate semialdehyde. Consequently, the NAD-dependent enzyme is present in cells grown on 4-aminobutyrate due to the formation of succinate semialdehyde. *E. coli* K-12, however, does not grow on hydroxyphenylacetate nor could mutants be selected which use it (86, 87). The NAD-dependent succinic semialdehyde dehydrogenase may result from the reactivation of a vestigial function lost by the wild-type *E. coli* K-12 strain unable to use 4-aminobutyrate and hydroxyphenylacetate.

The genes for arginine utilization in *E. coli* K-12 are scattered on the chromosome (19). The genes for biosynthetic arginine decarboxylase (*speA*) and for agmatinase (*speB*) are, however, closely linked at 64 min on the chromosome near the gene for biosynthetic ornithine decarboxylase (*speC*) (19). The genes for putrescine transaminase and 4-aminobutyraldehyde dehydrogenase were mapped at 89 and 30 min, respectively (19). The gene cluster *gabCPTD* involved in the utilization of 4-aminobutyrate was located at 57 min. These genes specify the following: *gabP*, the permease; *gabD*, the succinate semialdehyde dehydrogenase; *gabT*, the transaminase. *gabC* appears to code for a repressor controlling the synthesis of all three proteins (222, 223). According to Halpern's group, the genes *speA* and *speC* are constitutively expressed, while *speB* is induced by arginine or agmatine and is under carbon catabolite repression via a mechanism involving the cAMP-cAMP receptor protein complex. The enzymes of putrescine utilization appear subject to a glucose repression involving a cAMP-independent component (322). According to the same authors, the latter enzymes escape carbon catabolite repression under nitrogen limitation (322, 398). The results of Halpern's group are, however, in discordance with the data of the Satishchandran and Boyle groups (310, 390), who reported that the synthesis of arginine decarboxylase, ornithine decarboxylase, and agmatinase were enhanced by glucose and negatively controlled by the cAMP-cAMP receptor protein complex. The same authors found that the *speA* and *speC* enzymes were inducible by their respective substrates and repressible by putrescine. The discrepancy may be explained by the differences in strains used. As noted earlier, for instance, none of the *E. coli* K-12 strains used by the Boyle group was able to utilize agmatine as a nitrogen source, contrary to what was observed in other laboratories.

***Klebsiella* spp.** Unlike *E. coli* wild-type strains, *Klebsiella* spp. use arginine, agmatine, putrescine, and 4-aminobutyrate as carbon or nitrogen source or both (108, 109). No data are available on arginine decarboxylase in this organism. High levels of agmatinase are found in cells grown on arginine or agmatine as sole carbon and nitrogen source. However, in a ureaseless mutant, arginine utilization as sole carbon and nitrogen source is characterized by an excretion of urea corresponding to only 2% of substrate consumption as compared to 90 to 100% when agmatine is the sole carbon and nitrogen source. This indicates that arginine is not primarily used by the decarboxylase route but most likely via the arginine succinyltransferase pathway occurring in *Klebsiella* spp. Therefore, arginine utilization by the combined action of decarboxylase and agmatinase appears essentially directed towards polyamine synthesis. In line with this conclusion is the fact that arginine does not induce the subsequent enzymes of putrescine utilization as agmatine does; their syntheses are thus not coordinated with agmatinase, which is under the control of either arginine or agmatine.

Agmatine, putrescine, and 4-aminobutyrate induce formation of the four enzymes involved in putrescine utilization. The induction of these enzymes by agmatine is proably related to agmatine conversion into putrescine. This latter compound is more active than 4-aminobutyrate as inducer of putrescine transaminase and 4-aminobutyraldehyde dehydrogenase. As expected from their physiological role, the formation of these enzymes is, in addition to induction, subject to carbon and nitrogen catabolite repression.

***Pseudomonas* and *Aeromonas* spp.** In *Pseudomonas* and *Aeromonas* species arginine is converted into succinate in seven steps by arginine decarboxylase (step 1), agmatine deiminase, and *N*-carbamoylputrescine hydrolase (steps 2 and 3), and by enzymes of putrescine utilization: 4-aminobutyraldehyde is produced by either putrescine oxidase or putrescine transaminase (step 4) and is oxidized as in *E. coli* and *Klebsiella* spp. by a dehydrogenase (step 5). 4-Aminobutyrate transaminase and succinic semialdehyde dehydrogenase constitute the last steps (steps 6 and 7). This metabolism has been well documented in the various species, but information remains fragmentary for each individual strain.

Conversion of arginine into putrescine. Arginine decarboxylase is either inducible or constitutive according to strain. With strains able to use arginine as a carbon source by the succinyltransferase route (see above), an arginine-inducible arginine decarboxylase was noted (337) except for *P. cepacia*. In *P. syringae*, *P. stutzeri*, and in an unidentified

Pseudomonas species using arginine only as a nitrogen source (306, 337), arginine decarboxylase appears to be constitutive; in these organisms, as well as in *P. alcaligenes*, the decarboxylase route is the only arginine catabolic pathway. In the unidentified *Pseudomonas* sp. isolated by Rosenfeld and Roberts (306), the constitutive arginine decarboxylase is inhibited by its products agmatine, putrescine, and spermidine like the corresponding *E. coli* enzyme. In contrast, in *P. aeruginosa*, arginine elicits induction of the arginine decarboxylase; it is repressed by agmatine and its derivatives. This enzyme shows unusual properties: it is inhibited by agmatine, but its activity is stimulated by putrescine, spermidine, and spermine. These substances were also found to control negatively the OTCase belonging to the ADI pathway (see corresponding section). Polyamines may replace the Mg^{2+} ion required for full activity of the arginine decarboxylase. These molecules stabilize the quaternary structure of the enzyme (which dissociates upon dilution with loss of activity [A. Mercenier, Ph.D. thesis, University of Brussels, Brussels, Belgium, 1981]). By controlling in opposite fashion the ADI and arginine decarboxylase pathways, polyamines may serve as a signal for routing the metabolic flow from arginine along pathways whose physiological functions are different. For all *Pseudomonas* spp. so far tested, agmatine or carbamoylputrescine or both used either as sole carbon or nitrogen source elicit agmatine deiminase and *N*-carbamoylputrescine hydrolase synthesis, but little or no induction by arginine is noted for these enzymes (216, 337). They are repressed by putrescine and its derivatives (216). *Pseudomonas* strains able to use arginine as a carbon source have the arginine succinyltransferase pathway (see above) whose loss by mutation (in *P. aeruginosa*, for example) results in the ability to grow on arginine. Thus the arginine decarboxylase route serves mainly to produce polyamines when arginine is abundant. Supporting this view is the observation that *P. aeruginosa* mutants defective in agmatine deiminase or *N*-carbamoylputrescine hydrolase become unable to grow on agmatine and carbamoylputrescine as carbon and nitrogen sources but still retain their ability to grow on arginine as sole carbon and nitrogen source (130).

Enzymes of putrescine utilization. A transaminase, an oxidase, or both are involved in the formation of 4-aminobutyraldehyde. Putrescine transaminase activity is present in all *Pseudomonas* species (224). Putrescine transaminase and putrescine oxidase participate in putrescine catabolism in *P. fluorescens* and *P. aeruginosa* (224, 296). In *P. aeruginosa* and unidentified *Pseudomonas* species, putrescine transaminase and 4-aminobutyrate transaminase activities may be supported by the same enzyme molecule (44, 372, 393).

The synthesis of 4-aminobutyraldehyde dehydrogenase is induced under conditions of putrescine or pyrrolidine utilization and is repressed by the enzyme product 4-aminobutyrate (155, 225, 247). Mutants of *P. aeruginosa* defective in this enzyme become impaired in agmatine, carbamoylputrescine, and putrescine utilization; in addition, they do not grow on 2-ketoarginine. Therefore, a single aldehyde dehydrogenase seems to be involved in both guanidinobutyraldehyde and 4-aminobutyraldehyde oxidation in this organism (Haas, personal communication).

By contrast, distinct and separate aldehyde dehydrogenases are present in *P. putida* (Yorifugi, personal communication): an arginine-inducible, specific guanidinobutyraldehyde dehydrogenase and a putrescine-inducible 4-aminobutyraldehyde dehydrogenase showing a broad substrate specificity. The latter enzyme oxidizes guanidinobutyraldehyde, 5-aminovaleraldehyde, butyraldehyde, and propoinaldehyde in addition to 4-aminobutyraldehyde. Although these two dehydrogenases have the same subunit MW of 57,000, they differ in their quaternary structure: the guanidinobutyraldehyde-specific enzyme is a dimer, whereas the other enzyme is a tetramer (Yorifugi, personal communication).

The 4-aminobutyraldehyde dehydrogenase purified by Tchen and associates also shows a broad specificity since it oxidizes also 3-aminopropionaldehyde and succinic semialdehyde. It differs from the above-mentioned enzyme since it appears as a trimeric molecule (49) of subunit MW 75,000.

The next enzyme, 4-aminobutyrate transaminase, is induced by 4-aminobutyrate or its metabolic precursors (guanidinobutyrate, agmatine, carbamoylputrescine, and putrescine). Induction by arginine occurs in *P. putida* mainly via intermediates of the oxidase pathway and not via agmatine since *P. aeruginosa*, which does not have the oxidase pathway, displays only weak induction. The broad specificity of this enzyme allows it to participate in putrescine utilization (372, 383) and also in arginine biosynthesis: the uninduced level of this enzyme allows mutants of *P. aeruginosa* lacking acetylornithine aminotransferase to grow at a normal rate (372).

Certain *Pseudomonas* species contain both a NAD- and a NADP-specific succinic semialdehyde dehydrogenase; in addition, some of them have NAD-linked aminoaldehyde dehydrogenases with variable substrate specificities which are able to act on succinic semialdehyde (155, 238, 246, 254, 255). As carbon and nitrogen source 4-aminobutyrate induces the synthesis of a NADP-dependent succinate semialdehyde dehydrogenase in *P. fluorescens* ATCC 13430 (155) and *P. putida* IRC 204 (Legrain and Stalon, unpublished data) and of a NAD-specific enzyme in the *Pseudomonas* sp. isolated by Michaels and Tchen (225). The latter enzyme is composed of three subunits of MW 55,000 (304), which, in vivo, hybridize with the 75,000-MW subunit of either 4-aminobutyraldehyde dehydrogenase or 3-aminopropionaldehyde dehydrogenase, which are also trimeric proteins (49, 50). Immunological cross-reaction and hybridization between the last two enzymes suggest a close evolutionary relationship, but succinate semialdehyde dehydrogenase appears more distantly related since no immunological cross-reactions could be detected between this enzyme and the two previous ones (50, 305).

Other Pathways of Arginine, Citrulline, and Ornithine Utilization

Clostridia, which are obligate anaerobes, are able to utilize certain pairs of amino acids as energy sources by coupling the oxidation of one of them to the reduction of another one by the so-called Strickland reaction. In these organisms, ornithine may either be used as a hydrogen acceptor to produce 5-aminovalerate or an electron donor or it may be fermented as a single substrate.

Ornithine is produced from arginine by the ADI pathway in *C. botulinum* (227), *C. perfringens* (316), *C. sporogenes* (368), and other clostridia (331). The ability of *Clostridium* spp. to use arginine has been quoted also by Mead (211), who classified the clostridia into four groups according to amino acid utilization and fermentation end product. Organisms of group I produce 5-aminovalerate in arginine fermentation, group II utilizes arginine without producing 5-

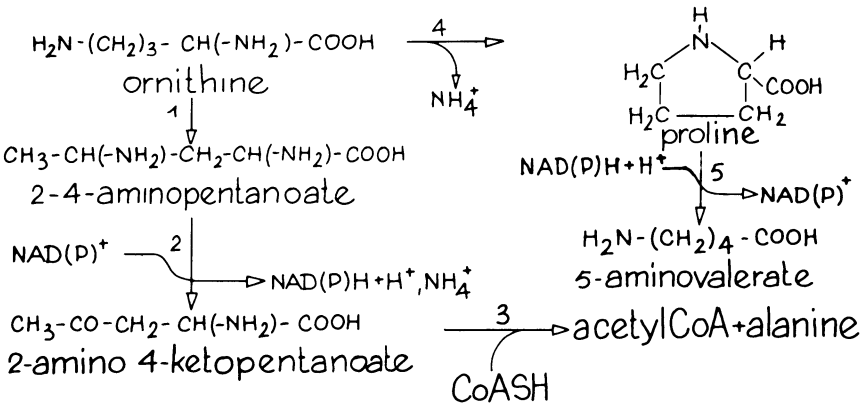

FIG. 8. Ornithine utilization by *Clostridium* spp. Steps: (1) D-ornithine-4,5-aminomutase, (2) 2,4-aminopentanoate dehydrogenase, (3) 2-amino-4-pentanoate CoA transferase, (4) ornithine cyclase, (5) D-proline reductase.

aminovalerate, group III is unable to utilize arginine, and group IV, consisting of one species, *C. putrefaciens*, utilizes it more slowly than groups I and II.

Ornithine oxidations is well documented. In *C. sticklandii*, ornithine oxidation starts with its isomerization by a racemase (352) followed by the migration of the amino group from C5 to C4, forming D-threo-2,4-aminopentanoate (93, 328, 352). This is oxidized to produce 2-amino-4-ketopentanoate and ammonia (329) (Fig. 8).

In a last step, 2-amino-4-ketopentanoate undergoes a thiolytic cleavage with CoA to form alanine and acetyl-CoA (157). The NADH produced during D-threo-2,4-aminopentanoate oxidation reduces D-proline to yield 5-aminovalerate (320). Proline racemase and D-proline reductase of *C. sticklandii* have been characterized (320, 332).

In *C. sticklandii* and *C. botulinum* the utilization of ornithine as a hydrogen acceptor involves the conversion of L-ornithine to proline by an ornithine cyclase (65, 66). The reaction proceeds by oxidation of ornithine to 2-ketopentanoic acid with the reduction of enzyme-bound NAD and release of ammonia. 2-Ketopentanoic acid undergoes ring closure to form 5-pyrroline-2-carboxylate, and the pyrrolidine ring is then reduced to form L-proline, using the enzyme-bound NADH as reactant (236). NAD proline reductase and 5-pyrroline-2-carboxylate dehydrogenase activities are found associated in *C. sporogenes* (332). The physiological role of these activities is thought to be the conversion of proline to glutamate (64).

Besides clostridia, ornithine cyclase has been found in aerobic *A. tumefaciens* (85), *P. cepacia*, and *P. putida* (Legrain and Stalon, unpublished data). In both *Agrobacterium* spp. and *P. putida* the enzyme is responsible for the dissimilation of the carbon skeleton since mutants of these organisms impaired in either ornithine cyclase or proline utilization cannot use ornithine as a carbon source.

In other *Pseudomonas* species, ornithine utilization was thought at first to occur by transamination with 2-ketoglutarate to produce glutamic semialdehyde since a transaminase activity is induced on ornithine-grown cells (63, 292, 371, 373). Besides, *P. aeruginosa* cells with a reduction in this activity were impaired in ornithine utilization (292, 372, 373). However, it is now clear that the transaminase in question is the succinylornithine transaminase involved in the arginine succinyltransferase pathway. Therefore, in this organism ornithine utilization first involves succinylation. Also, the arginine and ornithine succinyltransferase activities seem to be supported by the same protein molecule (Stalon, unpublished data).

An ornithine-specific succinyltransferase was found among some of the bacteria that possess the arginine succinyltransferase pathway, namely, *P. aeruginosa*, *P. aureofaciens*, *P. indigofera*, *P. doudoroffii*, *K. aerogenes*, and *Aeromonas formicans*. A very low level of this enzyme is found in *P. putida*, and no activity can be detected in *P. cepacia*. In these strains, however, ornithine catabolism occurs via proline formation catalyzed by an ornithine cyclase.

Citrulline utilization as a carbon source is restricted to relatively few species: *P. putida*, *P. indigofera*, and some *P. cepacia* strains (337). Mutants of *P. putida* impaired in ornithine cyclase activity fail to grow on citrulline, indicating that citrulline catabolism involves ornithine as an intermediate. Little is known of the conversion of citrulline into ornithine in these organisms. The catabolic OTCase belonging to the ADI pathway may fulfill this function in *P. putida*, the anabolic activity being repressed by citrulline and unable to catalyze in vitro the phosphorolysis of citrulline. In *P. cepacia* devoid of catabolic OTCase, the constitutive anabolic OTCase might catalyze the phosphorolysis of citrulline (Stalon, unpublished data). *P. indigofera*, when grown on citrulline, induces the arginine succinyltransferase and the enzymes associated with this route. Citrulline utilization in this bacterium, which lacks ornithine cyclase, may thus proceed via formation of arginine by the terminal step of the biosynthetic pathway. However, another possibility remains: conversion into ornithine and its utilization via the succinyl derivatives. The isolation of mutants in the various segments of this metabolism could solve this problem.

Bacteria with Multiple Pathways

The possession of different ways of dissimilating particular organic compounds as sources of carbon, nitrogen, or energy may have had a survival value in fluctuating environmental conditions. As a matter of fact, multiple arginine catabolic pathways frequently appear in the same bacterium, especially among members of the genus *Pseudomonas*. *P. putida* has four arginine catabolic pathways, while other *Pseudomonas* spp. of the fluorescent group and *Aeromonas* spp. have at least three ways of arginine dissimilation. Three routes have also been observed in cyanobacteria; at least two occur in the same organism: arginase and ADI, as in

some *Bacillus* strains. The roles of these divergent arginine catabolic pathways are largely independent. They are not used equally in all conditions and therefore there is little redundancy between them: in *Pseudomonas* spp. of the fluorescent group the ADI pathway serves to generate ATP under energy depletion conditions and the arginine succinyltransferase route is responsible for the dissimilation of the carbon skeleton in aerobic or anaerobic respiration, whereas the arginine decarboxylase route mainly meets polyamine requirements. An exception is *P. putida*, in which both the arginine succinyltransferase and the arginine oxidase routes are involved in the aerobic dissimilation of the arginine carbon skeleton.

Lack of redundancy also holds between the various catabolic pathways on one side and arginine biosynthesis on the other. Indeed, in the case of intermediates being common to a degradative route and to biosynthesis, regulatory mechanisms have evolved which prevent the operation of wasteful recycling by controlling OTCase activity (85, 150, 339; see above, arginine biosynthesis, OTCases, and CPSases). In the case of the succinyltransferase pathway, it is the succinyl "label" which plays that role.

EVOLUTIONARY CONSIDERATIONS

Biological Significance of Gene Organization

Are there constraints which define the map position of genes or is the topography we observe today largely, if not exclusively, the result of a series of historical accidents? Some constraints are likely to operate: e.g., the clustering near the *E. coli* replication origin of most of the genes required for macromolecular synthesis has been noted (20). In line with this observation, a correlation was observed (327) between the relative abundance of an amino acid in cell proteins and the position (distance from the origin) of the gene whose product was assumed to be rate limiting in the system (in this case the *argA* gene coding for the feedback-inhibited acetylglutamate synthetase). To strengthen the case, similar studies should be undertaken with a large number of bacteria.

In the particular instance of a scattered regulon such as in *E. coli*, it has been repeatedly emphasized that this type of topography (rather than the exact map position) allows finer tuning of gene control than clustering into a single operon. This consideration is of course much less relevant for those bacteria (such as *P. putida* and *P. aeruginosa*) in which there is little control of arginine biosynthetic genes.

There is indeed a marked lack of coordination between the individual repression responses of *E. coli arg* genes. The molecular basis of this pattern is becoming clear (see "Structure of control regions and the repression response"), but whether these widely different repression responses have a physiological significance remains a pending question. It could be advantageous or even necessary to the cell to adjust individually the rate of synthesis of enzymes working at metabolic branch points (such as the unique CPSase of *Enterobacteriaceae* and OTCase) or involved in both catabolism and anabolism (such as the succinylornithine aminotransferase of *Pseudomonas* spp.) or yet at steps using substrates available in limiting concentrations; there is, however, not enough quantitative evidence regarding metabolic fluxes on which to base a discussion of this topic. Besides, it is clear that, even within the *E. coli* species itself, different strains may display very different repression patterns: in *E. coli* B, which is weakly inducible by excess arginine but displays moderate derepression at low arginine concentrations, the intrinsically low level of enzyme makes control of the flux of arginine precursors by feedback inhibition no less efficient than in strain K-12 (99).

The naïve idea that an exclusive genetic pattern (not in terms of exact topography but in terms of grouping and resulting coordination) could be the preferred one for modulation of gene expression in the arginine regulon is already undermined by the variations encountered among the *Enterobacteriaceae*, in which *argG* is sometimes found clustered with *argE*, *-C*, *-B*, and *-H*. It becomes frankly inconsistent with the pattern found in *B. subtilis*; there, eight arginine biosynthetic genes, which are modulated over a wide range as in *E. coli*, are clustered in a 12-kilobase fragment. The evidence at hand (235) is not sufficient to estimate their degree of coordination, but at least the genes encoding OTCase (*argF*) and the arginine-specific CPSase are part of the same operon. *argG* and *argH* map outside the cluster but the data suggest that *argH* is coordinated with *argF* (135).

If one compares *B. subtilis* with *E. coli* it makes sense, of course, that the genes for the arginine-specific CPSase of the former are part of the cluster, whereas the unique and cumulatively repressed CPSase of *E. coli* is encoded by a separate locus.

The occurrence of divergent transcription from converging promoters such as in *argECBH* and *bio* also raises the question of the biological significance of this pattern. Does it reflect a physiological advantage, a type of rearrangement mechanism repeatedly used in evolution, or both? One obvious physiological advantage is that, from an internal control region, the whole operon is expressed more quickly than from one end. But then, why is the long *his* operon not organized in this way? On the other hand, rearrangements creating divergent operons (inverted repeats of *argE*) have been observed (58, 60). One could envision inverted duplications of whole operons, followed by selective inactivation and loss of duplicated genes on either side of the control region. Duplicative inversions caused by IS elements may also have been instrumental in the formation of divergent operons.

Deciding on the face of specific activities and sequence data whether different genetic arrangements and coordination patterns must be regarded as equally successful solutions arrived at under stringent selection or as merely tolerable ones will probably never be possible. This is, of course, a central question in many evolutionary debates. However, for microbes the tools are now available to deliberately engineer various types of arrangements and test their adequacy for survival over a large number of generations.

Lack of Attenuation Control

From the evidence discussed in the subsection, "Structure of control regions and repression response," in *E. coli*, it appears that a classical type of attenuation control is not used in the *arg* regulon of this organism. Since *argA* and *argS* are not yet sequenced, this statement remains provisional but will be taken as such in the following discussion. There are basically three ways of understanding a lack of attenuation control. (i) An attenuation mechanism based on arginyl-tRNA could be inefficient (discussed in reference 75), which is indeed not contradicted by the weak attenuation effect displayed by the *arg* codon present in the leader sequence of the *trp* operon (403). This view has the advantage of lending itself to experiments; it also provides an

incentive for the screening of other bacteria. (ii) Attenuation is absent in *E. coli* because, in this particular species (but maybe not in other ones) the arginine regulon never used this mechanism. (iii) It is absent today because it was lost, this loss having occurred at each locus individually or resulted (as in *E. coli*?) from scattering of a hypothetical ancestral arginine operon by chromosome rearrangements (see next section). The questions raised in (ii) and (iii) may, of course, never be answered; on the other hand, it is clear that the analysis of the arginine regulon in other bacteria will be highly relevant in this respect. Preliminary sequence data (Baumberg, unpublished data) suggest that attenuation is not used for the control of at least some *arg* genes in *B. subtilis*.

Chromosomal Rearrangements Involving Arginine Genes

Studies on rearrangements affecting the expression of arginine genes have led to original observations of general interest from the evolutionary point of view. Tandem duplications of *argE*, *argH*, or *argB* and *-H* were shown to occur in *recA* as well as in Rec^+ strains, calling attention to the possible role of illegitimate recombination between the two arms of a replication fork in this important type of evolutionary event (31). Subsequent work established that in Rec^+ strains, short tandem duplications (about 2 kilobases long) are, on a proportional basis, much more stable than expected from the rate at which longer duplications are lost (58). Duplications consisting of inverted repeats of *argE* arranged in divergent operons were also shown to occur spontaneously, forming single (and stable) or multiple (and unstable) palindromes (58, 60). High-copy-number amplification of *argF* was described recently (160); the occurrence of this rearrangement depends on the presence of an F factor integrated near the *argF* gene (161). The mechanism involved remains to be established: it appears to involve an "activation" of the IS*1* repeats flanking *argF*, followed by a *recA*-dependent amplification of the IS*1*-bordered fragment.

IS*2* elements integrated in the control region of a gene can turn it on constitutively (307). In the cases investigated up to now this "turning on" effect is due either to the association of a polymerase recognition site at extremity II of the element with a Pribnow box on the other side of the IS-chromosome junction (35, 116, 138, 156) or to DNA rearrangements creating a promoter near extremity I (113). In contrast, IS*3* was shown to carry a complete outward promoter able to reactivate a silent *argE* gene (59). A recent discussion of this topic may be found in reference 78.

Cryptic *argM* Gene of *E. coli*

The occurrence of cryptic genes in bacteria and the role their periodic activation may play in the evolution of bacterial populations have been reviewed recently (132). *E. coli argM* is a gene for an inducible but normally silent acetylornithine transaminase; it can be reactivated under selective pressure in *argD* strains (see "Enzymatic Steps and Regulation of Metabolic Flow"). The nature of this reactivation is presently under study.

That the *argM* protein is inducible by arginine, like the catabolic succinylornithine aminotransferase of *K. aerogenes*, and is similar to it in structure (107) suggests that *argM* is a cryptic element of a degradative pathway that is silent and possibly incomplete in current *E. coli* isolates. Active *argM* has, however, been selected for under conditions which did not imply a catabolic function but rather the transamination of glutamate with acetylglutamate semialdehyde to produce acetylornithine and 2-ketoglutarate. As the inducible succinylornithine aminotransferase from *Pseudomonas*, *Aeromonas*, or *Klebsiella* spp. (335), the *argM* product transaminates succinylornithine, displaying an even higher affinity for this substrate than for acetylornithine or ornithine (Stalon, unpublished observations). In the former organisms, the arginine-inducible succinylornithine transaminase is involved in the arginine succinyltransferase catabolic pathway (see above). It would therefore be interesting to see whether in *E. coli* successive mutations could not revive the very same pathway in its entirety. Since the *argM* protein is also able to transaminate ornithine to produce the semialdehyde of glutamate, another type of involvement in catabolism appears theoretically possible: *argM* might be part of a cryptic pathway leading from arginine to glutamate via ornithine. Clearly, much could be learned about metabolic evolution from further studies on this question.

Origin of CPSases

The properties of the microbial CPSases are typical of those of glutamine amidotransferases, a dozen enzymes displaying similar features (for reviews, see Buchanan [46], Meister [213], and a symposium on enzymes of glutamine metabolism [291]). (i) All can utilize ammonia as nitrogen donor in place of glutamine. (ii) All have separate binding sites for these two substrates, often located on distinct subunits. (iii) All have a glutamine binding site which contains a cysteine residue, never a serine, for example. (iv) All exhibit a glutaminase activity which is located on the same subunit as the glutamine binding site. Therefore, a common mechanism has been proposed to account for the use of glutamine by these various enzymes (197, 133, 350). It involves the covalent binding of glutamine to the cysteine residue in the glutamine binding site, the release of NH_3 from the amide group of glutamine, and its transfer, probably through a nonaqueous pocket, to an NH_3-binding site often located on a separate subunit where it is used for an amination reaction.

In view of these general properties of amidotransferases, it has been considered that these enzymes evolved by the combination of a common ancestral glutaminase with distinct ammonia-dependent aminases (197, 237, 290, 348). Evolution by duplication and mutation, and in some cases by gene fusion, of the gene for that ancestral glutaminase would thus be responsible for the various contemporary glutaminase genes. Substantial support for this evolutionary pathway was provided recently by the comparison of the primary sequences of the small subunits of *E. coli* and yeast CPSase to the glutaminase domains of other amidotransferases (102, 251, 277, 387). Both CPSases do indeed share significant homologies with the glutaminase components of anthranilate synthase of *E. coli* (245) and other sources (see references in reference 387) and of *p*-aminobenzoate synthase (167). Three highly conserved regions may be distinguished in these proteins and were recently found to be present also in the small component of *E. coli* guanosine monophosphate synthetase (399). The most highly conserved of these regions contains an invariant cysteine residue, which in the case of anthranilate synthase (168, 257) has been identified as the active site residue involved in glutamine binding. Interestingly, all three regions of homology are rich in hydrophobic

residues and might consequently contribute to the formation of the nonaqueous pocket necessary to protect NH_3 from protonation during its transfer to the NH_3-binding site. These various data strongly argue that the glutaminase subunits of CPSase, anthranilate synthase, *p*-aminobenzoate synthase, and guanosine monophosphate synthase were derived from a common ancestral glutaminase.

The elucidation of the primary sequence of both subunits of microbial CPSase has allowed us to address to the problem of the origin of CPSase I, the ammonia-dependent CPSase of mammalian liver. The idea that this acetylglutamate-requiring synthetase originated from the subsequent evolution of a glutamine-dependent enzyme was initially suggested by Trotta et al. (349, 350), who based their argument on MW comparisons. An elegant attempt to reconstruct the events involved in the emergence of the ornithine cycle from the enzymes of procaryotic metabolism of arginine has been presented by Paulus (261). This author has in particular analyzed the steps by which the actual ammonia-dependent CPSase may have evolved by fusion of the genes of the two subunits of an ancestral glutamine-dependent CPSase.

The complete sequence of rat CPSase I, as deduced recently from the sequence of the cognate mRNA, shows homology with the *E. coli* enzymes which encompasses the entire sequence of both the small and large subunits (248, 249). This confirms that the mitochondrial enzyme is indeed a hybrid polypeptide encoded by a gene resulting from the fusion of the two genes of the microbial enzymes. The glutamine domain of the microbial enzymes is still recognizable in the sequence of rat carbamoylphosphate synthetase, but the cysteine which is considered as participating in the binding of glutamine is replaced by a serine. Whether in the course of evolution this domain has retained a function in the binding of *N*-acetylglutamate, the effector of the mammalian enzyme, is unknown. However, this appears unlikely since carbamoylphosphate III of the teleost fish *Micropterus salmoides*, a synthetase which uses glutamine but requires acetylglutamate for activity, binds glutamine and *N*-acetylglutamate at separate but interacting sites (53). It will be of special interest to compare the amino acid sequence of this teleost enzyme with those of the microbial and mammalian liver CPSases.

Genetic Control of Carbamoylation: Origin of Carbamoyltransferases

A present, the most instructive evidence derived from studies of the *arg* genetic system and bearing on metabolic evolution concerns the genetic control of carbamoylation in *E. coli*, *Pseudomonas* spp., and a few other bacteria.

From the complete sequence of *F* and *I E. coli* OTCases (see reference 366) and of the catalytic subunit of ATCase (142), it is clear that the early suggestion based on structural considerations (189) and later supported by heteroduplex analysis (179) and partial sequencing data (115, 276) was correct: the two carbamoyltransferases are evolutionarily related. With an overall homology of 35 to 40%, concentrated in similarly structured domains (144, 366), the two enzymes provide a particularly eloquent case of enzyme differentiation following the duplication of an ancestral carbamoylase gene. The duplication may have occurred in tandem as suggested by the strong linkage existing between *argI* and *pyrB* in *E. coli* (152). The emergence of these two metabolic functions was probably a primordial event in metabolic evolution. If arginine was a "late intruder" in the genetic code, having replaced ornithine in the course of evolution (163), the OTCase gene may have been recruited from a gene encoding a substrate-ambiguous transferase primarily involved in the carbamoylation of aspartate.

The presence between two IS*1* elements of a duplicate OTCase gene (*argF*) in *E. coli* K-12 (145, 396) opens the possibility that this gene was translocated from a related species into *E. coli* K-12. This idea is supported by the fact that *argF* and *argI* exhibit greater amino acid than nucleotide homology, whereas, in general, the reverse obtains for duplicated genes in the same genome (300). Comparing homologous enzymes among *Enterobacteriaceae* renders the actual homology between *argF* and *argI* also consistent with this idea (366). However, these data alone cannot exclude the possibility that *argF* originated from *argI* by IS-promoted duplication in an ancestral *E. coli* cell, with possible interstrain transfer having taken place later on.

In *Pseudomonas* spp. possessing the ADI pathway, the biosynthesis and catabolism of citrulline are catalyzed by different proteins extremely specialized in their function since the anabolic OTCase is unable to catalyze the phosphorolysis of citrulline (due to the formation of an abortive citrulline-enzyme complex) (336), while the catabolic OTCase does not carbamoylate ornithine at phyisological concentrations of carbamoylphosphate (334, 339). Are these enzymes nevertheless related?

A study of the immunological relatedness between several catabolic OTCases from G^- and G^+ bacteria and some of their anabolic counterparts brings to light a surprising affinity between these catabolic enzymes and the *argF* OTCase of *E. coli*; in contrast, the same catabolic enzymes display little cross-reaction with anabolic OTCases from organisms other than *Enterobacteriaceae* (100). Limited sequence data are in keeping with this pattern: N-terminal amino acids from *argF* OTCase display, respectively, 45 and 28% homology with the corresponding residues of catabolic OTCases from *Aeromonas formicans* and *P. putida*. It is therefore possible that the *argF* enzyme is ancestrally related to some catabolic OTCase.

Despite their lack of immunological cross-reactivity, anabolic OTCases are probably related to each other also. Indeed, though the anabolic OTCases of *E. coli* and both *B. subtilis* and *B. licheniformis* share no antigenic determinants (100), a comparison between the respective amino acid sequences from *B. subtilis* (153 residues known) and *E. coli* discloses an overall homology of 32% (Baumberg, personal communication; R. Switzer, personal communication). We assume that most of the homologous residues are buried in the molecule.

Considering all these results together, it appears that both anabolic and catabolic OTCases on the one hand and the catalytic subunit of ATCase on the other share a common ancestral gene; this is also reflected in the sequence of yeast and human OTCases (Huygen, unpublished experiments, this laboratory; 143). The putrescine carbamoyltransferase functioning in the agmatine deiminase route of *Streptococcus faecalis* probably belongs to the same family since the N-terminal amino acid sequences of this enzyme and of catabolic OTCase from the same organism display 30 to 35% homology (P. Falmagne and V. Stalon, unpublished data). The agmatine deiminase route may represent an interesting case of evolution by pathway recruitment. The lack of immunological cross-reactivity between catabolic and anabolic OTCases in one and the same organism may at least in part be related to the necessity of not forming coaggregates which might be inactive.

Acquisition of the Ability to Use Anabolic or Catabolic OTCases in the Reverse Direction of Their Normal Operation

In the previous section we mentioned that the biosynthetic OTCases of *P. putida* and *P. aeruginosa* are kinetically unable to work in the reverse direction in vitro, while the catabolic enzyme cannot synthesize citrulline in vivo because of its extreme cooperativity towards carbamoylphosphate: mutants lacking the anabolic activity require citrulline or arginine for growth (124, 294). It is possible, however, to select for revertants in which the anabolic activity is still absent but the catabolic OTCase now fulfills the anabolic function. These revertants possess an altered catabolic OTCase with reduced or no cooperativity towards carbamoylphosphate (124, 339). The altered enzyme is kinetically very similar to *E. coli* OTCase. It is thus not unreasonable to see in the acquisition of a modified OTCase a reversal of the selection process that produced allosteric proteins with a quaternary structural design apt to generate extreme cooperativity. The catabolic OTCase of *Aeromonas formicans*, which is closely related antigenically to *E. coli* OTCase and to the catabolic enzyme of *Pseudomonas* spp., appears as an intermediate in this process. Indeed, this enzyme may occur as an active trimeric form like *E. coli* OTCase, but also as an active nonameric species (193). In *P. aeruginosa* the latter form is the only one to be active (Stalon, unpublished data). Therefore, in keeping with the data discussed in the preceding section, it appears that an ancestral OTCase gene may have duplicated itself and, under natural selection, produced the two metabolically specialized proteins we observe in today *Pseudomonas* spp.

Conversely, it is possible to reverse the metabolic role of an anabolic OTCase in vivo so as to use it for the synthesis of carbamoylphosphate. The enzyme itself is not modified, however, but its rate of synthesis is increased. For *E. coli* K-12, a mutant lacking CPSase requires both arginine and uracil for growth. Cells of this mutant grown in a medium containing citrulline can synthesize arginine by the final steps of arginine biosynthesis, but the only possibility they have to produce carbamoylphosphate for the synthesis of pyrimidines is phosphorolysis of citrulline. The wild type grows very slowly under these conditions, but faster-growing mutants display elevated levels of OTCase (192); these mutants express either *argF* or *argI* constitutively (as the result of operator mutations or gene amplification [160, 192]) or have a partial defect in argininosuccinate synthetase which allows citrulline to accumulate (192).

These metabolic reconversions illustrate the two main strategies of acquisitive metabolic evolution: direct alterations of the properties of an enzyme (in this case its allosteric properties) or regulatory mutations making it available in high amounts (62, 232).

Evolutionary Significance and Origin of Multiple Catabolic Pathways in *Pseudomonas* Species

The capacity of *Pseudomonas* spp. to synthesize the ADI pathway under oxygen limitation even in the absence of arginine may reflect the remnants of an ancient pathway having lost inductive control by arginine. It is obvious that in the course of evolution, microorganisms were faced with the problem of generating energy under anaerobic conditions. Possession of the ADI pathway may have been important in this respect, as attested to by its distribution among two major bacterial kingdoms, the archaebacteria (halobacteria) and five of the eight groups of the eubacterial line as defined by Fox et al. (106). Among these organisms the strictly aerobic *P. putida*, *P. fluorescens*, and *P. mendocina* appear as exceptions since they are able to ferment arginine but cannot use it for anaerobic growth. The ADI pathway may have a single evolutionary origin (at least in eubacteria) since the catabolic ornithine carbamoyltransferases of the various groups of organisms investigated display common antigenic determinants (see above). Such cross-reactions indicate a structural uniformity and suggest that there has been rather little evolution during the history of these enzymes. If we accept that the scheme of evolution began with the process of fermentation and progressed through a series of steps leading to the development of the contemporary aerobic organisms, the aerobic *Pseudomonas* spp. may then represent a form that lost its fermentative metabolism during the course of evolution.

The participation of molecular oxygen is required for the reaction catalyzed by arginine oxidase in *P. putida*. This activity is restricted to very few organisms and occurs in *P. putida* together with other arginine catabolic pathways. Yet, the mutational loss of this activity does not prevent the utilization of arginine as carbon source due to the presence of the succinyltransferase pathway. It is difficult to decide whether this enzyme is a recent acquisition or an ancient activity lost by the other *Pseudomonas* spp. during the course of evolution. However, the occurrence of this activity in the cyanobacterial group suggests that it arose soon after the appearance of oxygen on earth.

Does the possession of different pathways indicate a lack of evolutionary affinities? A comparison of *P. putida* and *P. aeruginosa* suggests that such an assumption is not valid. Indeed, both bacteria are physiologically related and bear strong homologies at the level of 16S ribosomal RNA. Both organisms use the arginine succinyltransferase, ADI, and arginine decarboxylase pathways. However, *P. aeruginosa* uses arginine as an energy source for anaerobic growth but *P. putida* does not; arginine oxidase is present in *P. putida* but is absent in *P. aeruginosa*.

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