

## Proline Excretion and Indirect Suppression in *Escherichia coli* and *Salmonella typhimurium*

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Received for publication 8 March 1974

The last step in proline biosynthesis in *Escherichia coli* K-12, *Salmonella typhimurium* LT7, and a number of other enterobacterial isolates is regulated so that no proline is excreted, even if excess  $\Delta^1$ -pyrroline-5-carboxylate, the immediate precursor of proline, is added to a culture. In proline auxotrophs blocked at an early step in proline biosynthesis (*proA* or *proB*), reversion to prototrophy is often due to a mutation in the arginine pathway which diverts *N*-acetyl glutamate  $\gamma$ -semialdehyde to proline synthesis, thus bypassing the *proA* or *proB* block. In such double mutants (*proAB*, *argD*), the last step in proline synthesis appears to be unregulated, since proline is excreted. Feedback inhibition and repression of the arginine pathway overcomes indirect suppression (restoring the *Pro*<sup>-</sup> phenotype), but proline regulation is not restored; double mutants still excrete proline when fed  $\Delta^1$ -pyrroline-5-carboxylate exogenously. A new class of proline analogue-resistant mutant, due to mutation at *argD*, is also described.

Proline biosynthesis in the enterobacteria proceeds via the following pathway: glutamate  $\rightarrow$  glutamate  $\gamma$ -semialdehyde  $\rightleftharpoons$   $\Delta^1$ -pyrroline-5-carboxylate acid (PCA)  $\rightarrow$  proline (2-4, 20, 23). The level of endogeneous proline is regulated by feedback inhibition and possibly also by repression (3-5).

Proline biosynthesis is normally well regulated, since wild-type strains of *Escherichia coli* and *Salmonella typhimurium* do not excrete PCA or proline. However, several classes of excreting mutants are known: *proC*<sup>-</sup> mutants which excrete PCA (23; L. J. Charamella and R. Curtiss III, Bacterial. Proc., p. 27, 1966), apparently because there is no endogenous proline to shut off the pathway; prototrophic proline-excreting regulatory mutants (4) which map at *proB* (5); *argD-proAB* double mutants (11) which had been reported to excrete PCA (12), although they actually excrete proline (see below); and *argD*<sup>-</sup> (*pro*<sup>+</sup>) single mutants which excrete proline (see below).

Two phenotypic classes of proline auxotrophs are distinguishable: *proC*<sup>-</sup>, which excretes PCA, and *proA*<sup>-</sup> and/or *proB*<sup>-</sup> (herein designated *proAB*<sup>-</sup>), which can grow on PCA. Revertants of any *proAB* mutant, including large deletion mutants, can be obtained (12). These revertants are not due to true back mutation, but rather to a mutation in the arginine pathway (*argD*) which restores proline

biosynthesis via an alternate, usually inoperative, pathway (D. F. Bacon and H. J. Vogel, Fed. Proc. 22:476, 1963; 11) (Fig. 1). *argD* mutants lack acetylornithine  $\gamma$ -transaminase, but are able to grow in the absence of arginine because a nonspecific transaminase substitutes for the missing enzyme (11). However, the resulting level of arginine synthesis is growth-limiting. *N*-acetylglutamate  $\gamma$ -semialdehyde accumulates behind the *argD* block and is deacetylated by the *argE* gene product to form glutamate  $\gamma$ -semialdehyde (11). Since the mutants have normal PCA reductase, glutamate  $\gamma$ -semialdehyde is converted to proline, bypassing the *proAB* block (Fig. 1). The phenotypic suppression of the requirement for proline in these strains is termed indirect suppression (8, 12). When exogenous arginine is present, arginine biosynthesis is repressed (9, 15), and as a consequence proline can no longer be synthesized via this pathway. Hence, indirectly suppressed mutants are proline-requiring in the presence of arginine.

In the absence of exogenous arginine, proline is synthesized in excess by the indirectly suppressed revertants and excreted into the medium (see below). In the presence of exogenous arginine, the indirectly suppressed revertants will excrete proline only if fed PCA. However, *argD*<sup>+</sup> strains will not excrete proline if fed PCA (in the presence or absence of arginine). The

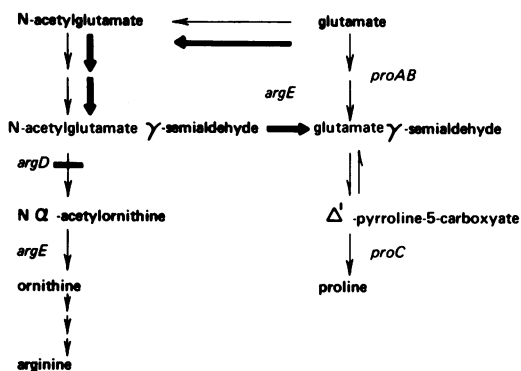


FIG. 1. The biosynthesis of arginine and proline from glutamate in enterobacterial strains. In *argD* mutants, the *argE* enzyme mediates the deacetylation of N-acetylglutamate- $\gamma$ -semialdehyde to glutamate- $\gamma$ -semialdehyde (11). Symbols:  $\rightarrow$ , normal pathways;  $\longrightarrow$ , *proAB* bypass pathway in *argD* mutants. *argD* (*argG* in *S. typhimurium*): acetylornithine- $\alpha$ -transaminase (EC 2.6.1.11). *argE*: acetylornithinase (EC 3.5.1.16). *proC*: pyrroline carboxylate reductase (EC 1.5.1.2).

physiological basis of this apparently pleiotropic effect of *argD* upon the regulation of proline synthesis or excretion is currently under investigation.

## MATERIALS AND METHODS

**Media.** The complete media used were L agar or L broth (13) supplemented with 10  $\mu$ g of thymine per ml. Vogel and Bonner medium E plus thymine and thiamine (MM) was employed as the minimal medium (16). MM+arginine (MM+a) contained MM plus L-arginine at 22  $\mu$ g/ml. Adenine at 20  $\mu$ g/ml was used where required. Arginine assay medium (AAM) contained MM supplemented with 1% Arginine Assay Medium (Difco). Streptomycin sulfate at 200  $\mu$ g/ml was used in transduction experiments.

**Chemicals.** PCA was provided by I. Shevchenko who synthesized it by the method of Strecker (19). (PCA is in spontaneous equilibrium with glutamate- $\gamma$ -semialdehyde.) Samples were neutralized before use with 5 M KOH. o-Aminobenzaldehyde (Sigma) was prepared as a 0.05 M solution in 20% ethyl alcohol and stored at 0°C. 3,4-Dihydro-DL-proline (DHP) (Calbiochem) was used at a final concentration of 1 to  $2 \times 10^{-5}$  M in distilled water. 4-Nitropyridine-N-oxide (4NPO) (K&K Laboratories) was used at a final concentration of 3 to 4  $\mu$ g/ml in distilled water. L-Azetidine-2-carboxylate acid (Sigma) was used at a final concentration of  $2 \times 10^{-5}$  M in distilled water.

**Strains.** The *E. coli* K-12 and *S. typhimurium* strains employed are listed in Table 1. Enterobacterial isolates from infected animals were provided by W. Parizek. All incubations were carried out at 37°C. The designation *proAB* is used for *proA* or *proB* mutants which have not been genetically distinguished. In addition, there is some indication that *proA* and *proB* do not comprise separate loci (M. Stodolsky, personal communication).

**Isolation of *proAB* auxotrophs.** Strain CB0401 has been described previously (16). Other *proAB* auxotrophs were isolated by penicillin (6) or 4NPO (10) enrichment. For 4NPO enrichment, single colonies of *E. coli* K-12 W3110 were incubated in AAM plus 4NPO until the cultures were turbid and then diluted  $10^{-4}$  and resuspended in AAM plus 4NPO. After a variable amount of time (1 to 3 days), most of the cultures were turbid and were again diluted into AAM plus 4NPO. After 3 to 4 cycles in AAM plus 4NPO, samples were plated on AAM and incubated. Colonies were replica plated onto MM, and presumptive auxotrophs were tested for their ability to grow on proline or PCA and for their crossfeeding phenotype.

**Selection of revertants.** Single colonies of strains CB0401, CB0437, CB0438, CB0439, or CB0440 were grown to stationary phase in L broth. Cells ( $10^7$  to  $10^9$ ) were plated on MM or MM+a and incubated for 48 h. The frequency of arginine-sensitive revertants was estimated by subtracting the number of revertants on MM+a from the number on MM or by replica plating from MM revertant plates to MM+a and MM.

After purification, revertants were tested for arginine sensitivity by spreading a culture on MM and spotting arginine and either PCA or proline about 3 cm apart on the plate. Indirectly suppressed revertants exhibit a zone of inhibition around the arginine spot which is overcome by proline or PCA.

**Selection of DHP-resistant mutants.** Single colonies of strain W3110 were grown to stationary phase in MM. A stationary culture (5 ml) was mixed with 25 ml of melted MM agar plus DHP. Plates were incubated for 24 to 48 h. Those colonies which had a halo of growth surrounding them were classified as excretors. Such colonies were purified and tested for DHP resistance in MM and MM+a.

**Growth measurements.** Single colonies of strain W3110 and of the *proAB-argD* double mutant growing in MM with aeration were resuspended in MM or in MM plus  $2 \times 10^{-5}$  M DHP and aerated. Changes in absorbance were measured at 420 nm in a Bausch & Lomb Spectronic 20 spectrophotometer.

**Biological test for PCA or proline excretion.** Single colonies of the strains to be tested were grown in L broth or appropriately supplemented MM. Cells were cross-streaked against *proAB* (which requires PCA or proline) and *proC* (which requires proline and excretes PCA) tester strains on MM and MM+a. Syntrophic growth was scored after 15 to 36 h of incubation.

**Chemical test for PCA excretion.** Strains were streaked on MM or MM+a and incubated for 24 to 48 h before 0.01 ml of 0.05 M o-aminobenzaldehyde was spotted adjacent to the streak. For tests in liquid medium 0.05 ml of a 0.05 M solution of o-aminobenzaldehyde was added to 5 ml of cells growing in MM or MM+a which had been incubated for 8 to 12 h. In each test, PCA excretion is indicated by a bright yellow color appearing within 15 min.

**Transduction.** Stocks of *Plkc* grown by the confluent plate lysis method on appropriate streptomycin-resistant (*str<sup>r</sup>*) donor strains were used to transduce streptomycin-sensitive (*str<sup>s</sup>*) recipients to *str<sup>r</sup>* (6). *str<sup>r</sup>* transductants were purified and checked for cotransduction of *argD*.

**Conjugation.** About  $10^6$  exponential phase donor cells per ml and about  $10^5$  recipient cells per ml were mixed in L broth and incubated 8 h without aeration. They were diluted 100-fold into fresh L broth and incubated overnight, before plating on MM supplemented with proline plus the amino acids required to support growth of the recipient, but not of the donor strain.

RESULTS

**Pro<sup>+</sup> revertants.** Pro<sup>+</sup> revertants were obtained from every *proAB*<sup>-</sup> strain of *E. coli* tested (Table 2). However, the frequency of revertants was considerably higher on MM than on MM+a. In addition, most revertant colonies appearing on MM, but not on MM+a, were surrounded by a halo of background growth. When revertant colonies from MM were purified by streaking out on L agar, most single colonies were still Pro<sup>-</sup> (75 to 90%), even when

care was taken to pick from the center of the revertant colony. Each revertant cell was apparently able to excrete sufficient PCA or proline to satisfy the growth requirement of several *pro*<sup>-</sup> cells. Consequently, each "revertant" colony was a mixture of auxotrophic and revertant cells. (Smith-Keary [17, 18] has reported even higher auxotroph frequencies in Pro<sup>+</sup> revertants of a *S. typhimurium proB* mutant.)

**Properties of revertants.** The indirectly suppressed revertants grew on MM, but were completely inhibited on MM supplemented with ornithine, citrulline, or arginine (Table 3), unless proline was also present. Similar results have been described for *S. typhimurium* by Kuo and Stocker (12).

Figure 2A is a photograph of a crossfeeding revertant strain (CB0436) streaked on MM against the parental *proAB*<sup>-</sup> mutant and a *proC*<sup>-</sup> tester strain. Since both auxotrophs were fed, it

TABLE 1. Bacterial strains

Strain	Relevant genotype	Source
<i>E. coli</i> K-12		
W3110		
CB0401	<i>proAB</i>	
CB0437	<i>proAB</i>	
CB0438	<i>proAB</i>	W3110
CB0439	<i>proAB</i>	
CB0440	<i>proAB</i>	
CB0441	<i>proC</i> <sup>-</sup>	W3110
CB0436	<i>proAB</i> <sup>-</sup> , <i>argD</i> <sup>-</sup>	CB0401
CB0442	<i>proAB</i> <sup>-</sup> , <i>argD</i> <sup>-</sup>	CB0437
CB0443	<i>argD</i> <sup>-</sup> , <i>str</i> <sup>r</sup>	W3110 ( <i>str</i> <sup>r</sup> )
CB0444	<i>recA</i> <sup>-</sup> , <i>proA</i> <sup>-</sup> , <i>argD</i> <sup>-</sup>	<sup>a</sup>
CB0445	<i>recA</i> <sup>-</sup> , <i>proA</i> <sup>-</sup> , <i>argD</i> <sup>-</sup> / <i>argD</i> <sup>+</sup>	KLF141 (14)xCB0444
<i>S. typhimurium</i> LT7		
3698	<i>proAB</i> <sup>-</sup>	Stocker (12)
3688	<i>proC</i> <sup>-</sup>	
3691	<i>proC</i> <sup>-</sup>	Stocker (12)
3710	<i>proAB</i> <sup>-</sup> , <i>argG</i> <sup>-</sup>	
3711	<i>proAB</i> <sup>-</sup> , <i>argG</i> <sup>-</sup>	
3714	<i>proAB</i> <sup>-</sup> , <i>argG</i> <sup>-</sup>	Stocker (12)
3791	<i>proAB</i> <sup>-</sup> , <i>argG</i> <sup>-</sup>	

<sup>a</sup> This strain was isolated as a Pro<sup>+</sup> revertant of a spontaneously cured (F<sup>-</sup>) KLF48/KL159 (14) derivative.

TABLE 2. Pro<sup>+</sup> revertants of *proAB*<sup>-</sup> *E. coli* K-12

Strain	No. of single colonies tested	Approx no. of cells plated (· 10 <sup>6</sup> )	Revertants <sup>a</sup>		
			No. on MM	No. on MM - a	Arginine-sensitive (%)
CB0401	13	6.7	56	2	96.4
CB0437	2	1.0	46	1	97.8
CB0438	5	2.7	265	57	78.4
CB0439	2	1.0	128	0	100.0
CB0440	2	1.0	150	2	98.7

<sup>a</sup> The frequency of revertants varied from single colony to single colony.

is apparent that the revertant excretes proline, not PCA. In addition, the *o*-aminobenzaldehyde color test (specific for PCA) on the revertant was negative, whereas the *proC*<sup>-</sup> tester was *o*-aminobenzaldehyde-positive. The feeding of *proAB*<sup>-</sup> by *proC*<sup>-</sup> (Fig. 2, bottom) provides an internal control that the correct strains were employed.

When a crossfeeding test was performed with the same strains on MM+a, the revertant colony did not grow, except where it was in juxtaposition with the *proC*<sup>-</sup> tester (Fig. 2B). However, as on MM (Fig. 2A), the *proC*<sup>-</sup> tester was able to grow in the vicinity of the revertant. The PCA excreted by the *proC*<sup>-</sup> tester had to have been converted to proline in the revertant and then excreted in order for the *proC*<sup>-</sup> strain to grow.

The crossfeeding results were reminiscent of those reported by Kuo and Stocker (12) with *S. typhimurium* LT7, except that Kuo and Stocker reported that their revertants excreted PCA, not proline. We repeated the crossfeeding tests with several of Kuo and Stocker's strains and found exactly the same pattern of crossfeeding and *o*-aminobenzaldehyde reaction as with the K-12 strains (data not shown). Therefore, under our conditions at least, indirectly suppressed revertants of *S. typhimurium* also excrete proline, but not PCA.

*argD* is cotransducible with *strA* (21). To confirm that the second genetic lesion in the indirectly suppressed revertants is due to mutation at *argD*, a *str*<sup>r</sup> isolate of the double mutant strain (CB0436) was obtained and used as the transductional donor with the parental *proAB*<sup>-</sup> mutant strain (CB0401). Out of over 100 *str*<sup>r</sup> transductants tested, all were Pro<sup>+</sup> and excreted proline on MM, but were Pro<sup>-</sup> on MM+a.

We did notice differences in growth rate and arginine sensitivity between indirectly suppressed revertants in *E. coli* and *S. typhimurium*. The *E. coli* strains grew better than the *S. typhimurium* strains in the absence of arginine. This may reflect a higher endogenous activity of the nonspecific transaminases in *E. coli* which can substitute for the *argD* gene product. In addition, the *S. typhimurium* strains were not inhibited by arginine, probably because arginine regulatory mutants have a selective advantage in these strains (11) and may have arisen since the strains were initially studied.

**Syntrophism involving *argD*<sup>+</sup> strains.** Figures 2C and D illustrate the pattern of crossfeeding obtained with the parental *pro*<sup>+</sup> strain (W3110). Neither tester strain was stimulated

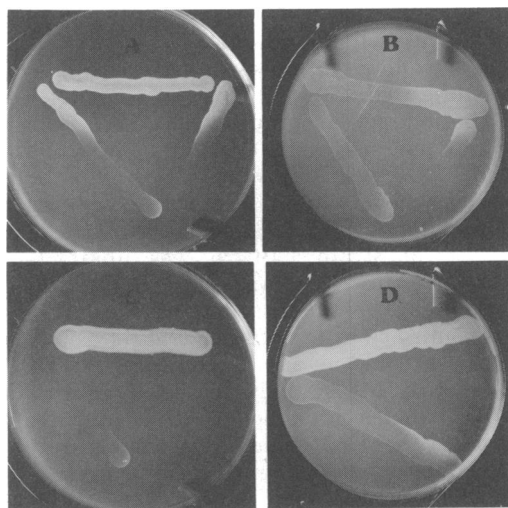


FIG. 2. Syntrophism tests with *E. coli* K-12 on MM (A, C) and MM+a (B, D). A and B: CB0436, *proAB*<sup>-</sup>, *argD*<sup>-</sup> (top); CB0438, *proAB*<sup>-</sup> (left); and CB0441, *proC*<sup>-</sup> (right). C and D: W3110 (top); CB0438, *proAB*<sup>-</sup> (left); and CB0441, *proC*<sup>-</sup> (right). There is more background growth on plates B and D because they were incubated for 36 h, whereas plates A and C were incubated for 22 h.

by strain W3110. Therefore, we can conclude that strain W3110 does not excrete either proline or PCA on MM or on MM+a. Even when fed PCA syntrophically (by the *proC*<sup>-</sup> tester), strain W3110 still does not excrete proline, in contrast to the indirectly suppressed revertants. This observation is contrary to expectation, since Baich and Pierson (4) had reported that *E. coli* W excretes proline when fed PCA.

We have tested strains of *E. coli* B, C, and W from the laboratory collection and another 19 enterobacterial strains recently collected from diseased animals. *E. coli* strains B, C, and W and 17 of the new isolates did not crossfeed the *proAB*<sup>-</sup> or *proC*<sup>-</sup> testers on MM, and two of the new isolates fed both. Proline is, therefore, not excreted by the majority of enterobacterial isolates.

The possibility that *argD*<sup>+</sup> strains do not excrete proline because proline is degraded efficiently within the cell was tested by examining the crossfeeding behavior of *pro*<sup>+</sup> strains defective in proline oxidase (the first step in proline catabolism) or in PCA dehydrogenase (the second step) (Rossi et al., unpublished data). Neither strain excreted proline, even when fed PCA, indicating that catabolism is probably not involved in the regulation of proline excretion.

**Resistance to DHP.** Mutants resistant to the

proline analogue DHP are defective in either the uptake of proline (and DHP) or in the regulation of proline synthesis, so that excess proline is synthesized (22).

The indirectly suppressed revertants, which excrete proline (Fig. 2A), should be resistant to DHP. To test this hypothesis, a drop of DHP was spotted onto a MM plate spread with strain CB0436 (*argD*<sup>-</sup>, *proAB*<sup>-</sup>) or strain W3110. The growth of strain CB0436 was unaffected, whereas strain W3110 was completely inhibited in the vicinity of the spot (data not shown). This was confirmed by following the rate of growth in the presence and absence of DHP (Fig. 3). DHP ( $2 \times 10^{-5}$  M) severely inhibited growth of W3110, whereas it did not affect the growth rate of strain CB0436. Strain CB0436 is also resistant to azetidine-2-carboxylic acid, another proline analogue (data not shown).

DHP-resistant colonies were isolated directly by pour plating strain W3110 (*str*<sup>r</sup>) on MM plus DHP. Some colonies had halos of satellite growth around them (indicative of proline excretion) and other (presumptive permease mutants) did not.

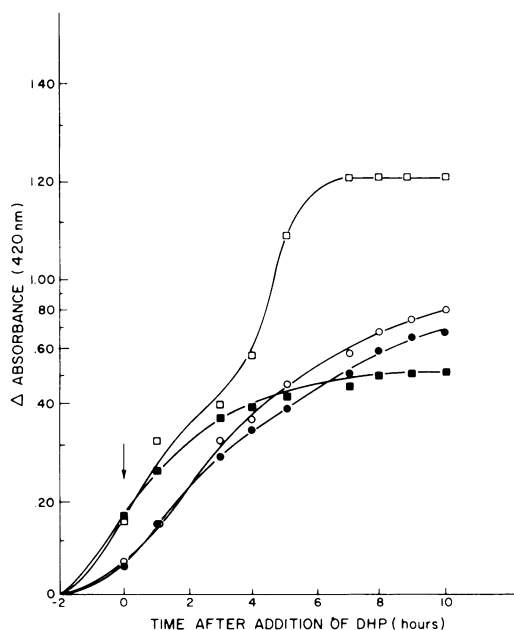


FIG. 3. Growth of W3110 and CB0436 in the presence and absence of  $2 \times 10^{-5}$  M DHP. DHP was added at 0 h (↓). Symbols: □, W3110; ■, W3110 plus DHP; ○, CB0436; ●, CB0436 plus DHP. CB0436 grows more slowly than W3110 because it, like similar *S. typhimurium* strains (12), has a partial requirement for arginine. In medium containing arginine and proline, CB0436 grows at wild-type rate (not shown). An absorbance of 0.6 is equivalent to 0.84 mg/ml dry weight.

Twenty-five haloed colonies were purified and tested for proline excretion and DHP sensitivity in the presence and absence of arginine. Most were proline-excreting and DHP-resistant on both MM and MM + a. These were probably *proB* regulatory mutants similar to those previously described (4, 5). One colony, however, was DHP<sup>r</sup> in the absence of arginine, but sensitive to DHP in the presence of arginine. This is the phenotype expected of a mutant which is resistant to DHP as a result of a block at *argD*.

In an experiment in which this strain was used as a transductional donor to W3110, about 5% of the *str*<sup>r</sup> recipients (on MM) were also DHP<sup>r</sup>, indicating that the loci are linked. The relatively low cotransduction frequency was not investigated further.

**ArgD<sup>-</sup>/argD<sup>+</sup> heterogenotes.** Eight exconjugants from the cross CB0444 (*argD*<sup>-</sup>, *proA*<sup>-</sup>) × KLF141 (*argD*<sup>+</sup>/*argD*<sup>+</sup>) were tested for proline auxotrophy and excretion. All were Pro<sup>-</sup> (in the presence or absence of arginine) and failed to excrete proline, even when fed PCA. Spontaneous segregants obtained from broth-grown cultures of three of these exconjugants behaved like the indirectly suppressed recipient (Table 3).

The observation that the exconjugants had the same phenotype as the proline auxotrophs indicates that the mutation responsible for indirect suppression (*argD*<sup>-</sup>) is recessive.

## DISCUSSION

The finding that most revertants of all of the *proAB*<sup>-</sup> mutants studied were extragenic (indirectly suppressed), not intragenic (Table 2), was unexpected, since in general revertants arise by mutation within the same rather than a different gene. Part of the explanation may reside in the nature of the genetic changes which will give rise to a revertant. Whereas intragenic revertants must have restored enzyme activity and, hence, can arise by mutation at only one or a few sites in the mutant gene, extragenic revertants of *proAB* mutants need only lose *argD* function by mutation at any of a number of sites in *argD*. It is also possible that extragenic revertants are more apt to survive and produce a visible colony on crowded reversion plates than are intragenic revertants of *proAB* mutants. Since indirectly suppressed revertants excrete proline and stimulate their neighboring nonrevertant cells to grow, large syntrophic colonies are formed. The microecological conditions which are very important for the growth of revertants or prototrophs on crowded plates have not been studied in the present system. Indirectly suppressed revert-

TABLE 3. Properties of *E. coli* K-12 and *S. typhimurium* LT7 strains

Relevant genotype			Pro phenotype		Excretion		
<i>proAB</i>	<i>proC</i>	<i>argD</i> <sup>a</sup>	On MM	On MM + a	On MM	On MM + a	On MM + a plus PCA
+	+	+	+	+	-	-	-
+	-	+	-	-	PCA	PCA	-
-	+	+	-	-	-	-	-
-	+	-	+	-	Proline	- <sup>b</sup>	Proline
-	+	-/+	-	-	-	-	-
+	+	-	+	+	Proline	-	Proline

<sup>a</sup> *argD* is equivalent to *argG* in *S. typhimurium*.

<sup>b</sup> The *S. typhimurium* strains had lost their arginine sensitivity (probably due to arginine regulatory mutations) and excreted proline.

ants can, however, be completely eliminated from the revertant population by plating on medium containing arginine. It is possible that more subtle changes in the medium or plating conditions could drastically affect the frequencies of the various types of revertants. There has been one careful study of Pro<sup>+</sup> revertant types and of the homogeneity of revertant colonies (17). However, it is difficult to assess that work because the author was unaware of indirect suppression by *argD* mutants and invoked "plasmid-linked suppressor mutations" (18) to account for his results.

Our observation that indirectly suppressed revertants of *proAB* mutants excrete proline in minimal medium, and Tristram and Thurston's report (22) that proline overproduction is the basis of one class of analogue-resistant mutant led us to test the resistance of these revertants to DHP. We found that they were resistant to DHP in the absence of arginine, but normally sensitive to DHP in the presence of arginine. These observations led to the hypothesis that among DHP<sup>r</sup> mutants of a *pro*<sup>+</sup> strain we might find mutants which had a normal proline pathway, but which were *argD*<sup>-</sup>. Such a mutant was found, and, as expected, was proline-excreting and DHP<sup>r</sup> in the absence of exogenous arginine, but DHP<sup>s</sup> in the presence of arginine (not shown). This mutant represents a new class of analogue-resistant mutant. Proline analogue resistance provides an additional method for isolating *argD* mutants which are difficult to obtain directly (9, 15), because the *argD* gene product is not essential for growth (11) (Fig. 2A, 3).

The ability of indirectly suppressed revertants to excrete proline when fed exogenous PCA was first observed on crossfeeding test plates containing arginine. On such plates neither *proC*<sup>-</sup> nor indirectly suppressed *proAB*<sup>-</sup> strains can grow. However, we observed that both strains grew where they were in juxtaposition to

each other (Fig. 2B). It was apparent that the PCA excreted by the *proC*<sup>-</sup> strain permitted the indirectly suppressed revertant to grow and excrete proline which, in turn, fed the *proC*<sup>-</sup> strain. This is an example of mutual syntrophism (7) of a particularly intimate type. The mutants which feed each other are blocked in adjacent steps in the same biosynthetic pathway.

The final step in proline biosynthesis, the conversion of PCA to proline, is in some cases unregulated (4, 5). (i) We found (see above), as did Baich and Pierson (4), that several enterobacterial isolates excrete proline when fed PCA; (ii) Baich and Pierson were also able to obtain proline-overproducing mutants (affecting the first proline biosynthetic step), which were no longer feedback inhibitable; (iii) Condamine (5) found that similar proline-overproducing mutants in *E. coli* K-12 are regulatory mutants mapping at *proB*; and (iv) we found that both *argD*<sup>-</sup> strains of *E. coli* K-12 and indirectly suppressed (*argD*<sup>-</sup>) revertants of *proAB*<sup>-</sup> strains of both *E. coli* K-12 and *S. typhimurium* LT7 excrete proline on MM and also on MM+a when supplied with exogenous PCA (Table 3; Fig. 2B). However, there is contradictory evidence suggesting that the final step is regulated: (i) Kuo and Stocker (12) reported that indirectly suppressed revertants of *S. typhimurium* LT7 excrete PCA, not proline; and (ii) we were unable to detect any proline excretion when wild-type or *proAB*<sup>-</sup> strains of *E. coli* K-12, or *proAB*<sup>-</sup> strains of *S. typhimurium* LT7, were fed excess PCA.

Upon reexamination of the indirectly suppressed revertants studied by Kuo and Stocker (12), we found that they excreted proline, not PCA. Hence, regulation at the final step is only clearly implicated by our observation that there is no proline excretion in *E. coli* K-12, *S. typhimurium* LT7, and most enterobacterial isolates tested, even when they are fed PCA.

However, the proline-overproducing mutants which we, Baich and Pierson (4), and Condamine (5) isolated on the basis of analogue resistance would not have been isolated if they excreted PCA instead of proline. Therefore, these strains might be double mutants, one mutation affecting *proC* regulation and the other *proB* or *argD*.

The loss of *proC* regulation in indirectly suppressed *proAB* revertants must also be explained. Although it is possible that this is due to incomplete feedback inhibition and repression of the arginine pathway, this is not likely because the proline requirement of the indirectly suppressed revertant is absolute in the presence of arginine, whereas leak-through should eventually result in detectable growth.

Although we have not yet compared the *proC* enzyme (PCA reductase) from *argD*<sup>+</sup> and *argD*<sup>-</sup> strains, our observations that *argD*<sup>-</sup>/*argD*<sup>+</sup> heterogenotes of a *proA*<sup>-</sup> strain are Pro<sup>-</sup> and do not exhibit mutual syntrophism suggest that the *argD* mutation itself has a pleiotropic effect upon *proC* regulation.

#### ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-10862 from the National Institutes of Allergy and Infectious Diseases.

We thank W. Parizek and B. A. D. Stocker for the gift of bacterial strains, I. Shevchenko for the gift of PCA, M. Stodolsky for unpublished information, R. Vinopal and J. Coleman for helpful discussions and reviews of the manuscript, and M. Conlon for assistance in some experiments.

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