

# SPONTANEOUS AND MUTATOR-INDUCED REVERSIONS OF AN *ESCHERICHIA COLI* AUXOTROPH

## I. PROTOTROPHIC TYPES AND THEIR GROWTH CHARACTERISTICS<sup>1</sup>

DONALD F. BACON<sup>2</sup> AND HENRY P. TREFFERS

*Department of Microbiology, Yale University, New Haven, Connecticut*

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A profitable experimental approach to the problem of the nature of genic control is the study of independent reversions of nutritional mutants to forms resembling the wild type (prototrophs). This has been ably demonstrated by Giles (1951, 1956, 1958), Giles, Partridge, and Nelson (1957), Yanofsky and Bonner (1955), Pateman (1957), and Fincham (1957) with *Neurospora crassa*.

Davis (1950a) reported that among a dozen spontaneous and ultraviolet-induced reversions from a *p*-aminobenzoic acid requirement to *p*-aminobenzoic acid independence in a stock culture of *Escherichia coli*, six different rates of growth in the absence of *p*-aminobenzoic acid could be recognized. Some of the revertants with growth rates slower than that of the wild type had their growth rates restored by *p*-aminobenzoic acid, others did not. In the following year Bryson (1951) reported that revertants of histidine- and methionine-requiring mutants of *E. coli* frequently differed from the original wild type as judged by their reduced growth rate and reduced final titer when grown in minimal media.

In these and subsequent reports there has been increasing evidence that changes to prototrophy in auxotrophic mutants may represent a variety of different mutational events which, in turn, give rise to a variety of different prototrophic types. The present paper reports the occurrence of several revertant types following instances of spontaneous reversions from an ornithine-requiring state to ornithine independence in *E.*

*coli*. The growth characteristics of some of these revertants will be described.

### MATERIALS AND METHODS

*Organisms.* The cultures of *E. coli* used in this study were: the wild type of strain W (ATCC 9637); an ornithine-requiring mutant, 160-37, derived from strain W (Vogel, 1953); a derivative of 160-37 which has an additional requirement for proline, 160-37D2 (Vogel, 1953); and an *E. coli* K-12 strain which possesses the mutator gene (M\*) and has requirements for biotin and phenylalanine, 58-278M\* (Treffers, Spinelli, and Belser, 1954).

*Culture media.* The minimal medium of Davis (1950b) was modified slightly by increasing the glucose content to 0.2%. Minimal agar was prepared by mixing double strength minimal broth with an equal volume of a hot sterile 3% solution of agar. Ornithine hydrochloride was dissolved in distilled water at a concentration of 10 mg per ml and sterilized by filtration; this stock was used to supplement minimal media as detailed in the text. Brain heart infusion broth was used as a complete medium.

*Photometric measurements of growth.* Experiments in which the growth of various stock cultures in minimal broth was followed photometrically were of two forms: in the earlier experiments 0.1-ml amounts of overnight cultures in minimal broth, supplemented with 10 µg of ornithine·HCl per ml when necessary, were inoculated into 10 ml of the appropriate broth medium. Samples of these cultures were added to sterile flat-bottomed, optically uniform tubes which contained small glass-covered magnets. These cultures, together with similar uninoculated tubes as media controls were set up in a special recording turbidimeter (Rubin, 1950).

In later experiments the cells were to be harvested for enzyme studies and the growth in

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<sup>2</sup> Present address: Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey.

200-ml broth cultures was followed by removing samples and reading their densities photometrically. The cultures were maintained at 37 C and shaken in a reciprocating shaker at approximately 60 oscillations per min. As growth became apparent, 10-ml samples were removed and the optical densities read in a Coleman nephelometer, model no. 9, using light filter 8-215 which has maximal light transmission at 655 m $\mu$  wavelength. The terms "turbidimeter" or "colorimeter" will be used to differentiate the instrument and the experimental conditions which were employed.

*Conjugation between strain W and K-12.* The mutator gene present in culture 58-278M\* of strain K-12 was introduced into mutant 160-37D2 of *E. coli* strain W by conjugation; this latter organism served as the recipient, or F<sup>-</sup> mating type (Lederberg, Cavalli, and Lederberg, 1952; Hayes, 1953). Samples (1 ml) of overnight cultures in brain heart broth were centrifuged and resuspended in 2 ml of fresh brain heart broth. To 0.6 ml of the F<sup>-</sup> (160-37D2) cell suspension was added 0.2 ml of the F<sup>+</sup> (58-278M\*) broth suspension. This mixture was shaken gently at 37 C for 45 min; then it was centrifuged and the pellet washed once in 1 ml of sterile saline and finally resuspended in 2 ml of saline. Volumes (0.1 ml) of this final suspension were spread on minimal agar plates supplemented with ornithine·HCl at a level of 10  $\mu$ g per ml, thus selecting for loss of the proline requirement. These recombinants were subsequently screened for the presence of mutator gene.

*Screening of recombinants for presence of mutator gene.* Recombinant colonies were streaked onto a complete medium and after incubation, an isolated colony from each culture was inoculated into 2 ml of brain heart broth; after overnight incubation at 37 C, 0.1 ml from the fully grown broth cultures was spread on brain heart agar which contained 100  $\mu$ g of dihydrostreptomycin sulfate per ml. These plates were incubated for 24 hr and then scored for colonies. Those recombinants which repeatedly gave rise to more than 10 resistant colonies were regarded as possessing the mutator gene (Treffers et al., 1954; Skaar, 1956).

#### RESULTS

Mutant 160-37 lacks the enzyme acetylornithinase (Vogel, 1953) and thus requires an exogenous

source, either of ornithine or arginine for growth. It was observed that not all revertants from this requirement had the same colonial characteristics as the wild type when these various prototrophs were grown on minimal agar.

Spontaneous reversions from the auxotrophic requirement were therefore screened for different revertant types: an overnight culture of mutant 160-37 in brain heart broth was diluted 10<sup>-6</sup> in saline and 1 ml of this dilution added to 100 ml of minimal broth supplemented with 20  $\mu$ g of ornithine·HCl. This inoculated medium was dispensed in 4-ml amounts in  $\frac{3}{4}$ -in. test tubes which were shaken vigorously at 37 C overnight. The cultures were then centrifuged and the pellets were resuspended in minimal amounts of saline and transferred to minimal agar plates which were incubated at 37 C in a moist chamber. These plate cultures were examined daily for 10 days and as the colonies appeared they were streaked onto minimal agar; the latter subinoculations made it possible to score for the colonial growth characteristics, that is, revertant type. The time of appearance of prototrophic colonies on the original plate could not be relied upon as a means of classifying the revertants; colonies which were subsequently scored as representing fast growing revertants appeared on the original plates as late as 96 hr, although the expected time at which they should have appeared was 24 hr. This late appearance of prototrophic colonies will be the subject of a separate communication.

When the mutants were screened in the above manner they were observed to be of four major types:

F (fast) (these resemble the wild type): large colonies on minimal agar after 24 hr at 37 C.

S48 (slow, 48 hr): large colonies on minimal agar after 48 hr at 37 C.

S72 (slow, 72 hr): large colonies on minimal agar after 72 hr at 37 C.

VS (very slow): small colonies on minimal agar after 5 to 7 days at 37 C.

As was noted, in the conjugation procedure selection was made for proline independence; of these recombinant colonies, 6 of 242, or 2.5% were also ornithine independent (pro<sup>+</sup>orn<sup>+</sup>) and 17 of 104, or 16.3% were positive when screened for the presence of the mutator gene. One of these mutator-containing exconjugants (Z2/9) was examined by the negative tube method (Luria and Delbrück, 1943; Newcombe, 1948)

for its frequency of reversion to ornithine independence; the mean of 10 determinations was  $3.76 \times 10^{-7}$  per bacterium, per generation, with a standard error of  $0.43 \times 10^{-7}$ . For the nonmutator stock culture (160-37), the mean of 12 determinations was  $1.16 \times 10^{-10}$  per bacterium, per generation, with a standard error of  $0.16 \times 10^{-10}$ . Thus, the increase in rate of reversion in the presence of the mutator gene was  $3.2 \times 10^3$  or 3,200-fold.

The mutator-induced revertants were also screened for different revertant types. A small number of cells from culture Z2/9 was inoculated into minimal broth supplemented with 0.1  $\mu$ g of ornithine·HCl per ml and from this, 0.1-ml amounts were taken up in sterile, graduated pipettes; the cultures contained in these pipettes were incubated at 37 C in a moist chamber. This level of ornithine limited the final number of auxotrophic forms obtained and, therefore, the number of reversions, to a convenient level. Shortly after growth of the auxotrophic forms had ceased, due to exhaustion of the limiting amount of ornithine, each 0.1-ml culture was spread over the surface of a minimal agar plate and the latter incubated at 37 C in a moist chamber. The majority of plates developed from one to three prototrophic colonies; a total of 320 appeared on 200 such plates. Each colony was restreaked on minimal agar and it is noteworthy that in all cases these colonies were scored as representing S48 revertants.

Of the slower growing prototrophs derived from the nonmutator culture 160-37, both the VS and S72 types readily "throw off" faster growing forms, but to date the VS revertants have been given only a limited amount of attention. The readiness with which S72 revertants throw off S48 types is characteristic of these prototrophs. The conversion, however, of an S48 to an F revertant is a much rarer event, although under conditions of repeated subinoculation of S48 prototrophs in minimal broth these cultures are eventually overgrown by F revertant types.

An attempt was made to measure the frequency with which S72 revertants give rise to S48 cells. As will be evident from data to be presented in more detail below, all of the four revertant classes had approximately the same rate of growth in minimal medium supplemented with ornithine. Thus, S48 cells arising in an S72 revertant culture would be expected to have a minimal selective

TABLE 1  
*Number of S48 colonies, representing S48 cells, present in the 0.1-ml cultures of S72 revertants at time of plating*

Mutant	Culture No.	No. of S48 Colonies
22A	1	11
	2	4
	3	3
	4	4
	5	6
	6	4
	7	17
	8	6
	9	17
	10	6
	11	10
	12	15
	13	17
	14	16
	15	11
	16	11
	17	5
	18	16
	19	35
	20	12
34	1	19
	2	4
	3	8
	4	9
	5	10
	6	15
	7	24
	8	18
	9	7
	10	20
	11	9
	12	12
	13	15
	14	12
	15	26
	16	9
	17	94
	18	14
	19	14
	20	13

advantage if sufficient ornithine were present. Two S72 prototrophs designated 22A and 34, of independent origin, were plated on minimal agar and in each case a single colony was picked into 5 ml of minimal broth supplemented with 10  $\mu$ g of ornithine·HCl per ml. These cultures were

incubated at 37 C for 24 hr and at that time 0.1 ml of a  $10^{-5}$  dilution of the broth cultures was added to 10 ml of minimal broth also supplemented with 10  $\mu\text{g}$  of ornithine per ml; 0.1-ml amounts of this inoculated broth were taken up in 23 sterile pipettes and incubated at 37 C in a moist chamber for 20 hr. The probability of including a "preformed" S48 cell in the inocula for each set of pipettes was estimated from control plates; the highest estimate was one S48 cell per 33 pipettes. Furthermore, such an event would be fortuitous, that is, result in an inordinate number of colonies on one plate.

After 20 hr of incubation, the contents of each of 20 pipettes (0.1 ml) were spread on minimal agar plates and reincubated; 3 additional pipettes were assayed on brain heart agar.

The mean viable count of mutant 22A was  $0.9 \times 10^8$  cells per 0.1-ml culture and that of mutant 34 was  $0.8 \times 10^8$  cells per 0.1-ml culture. After 48 hr of incubation, the minimal agar plates were scored for S48 colonies (Table 1).

For the most part, when the plates were scored for S48 colonies, the results seemed unambiguous; a burst of colonies which had first become visible at about 30 hr was clearly apparent at 48 hr and these were scored as representing S48 cells present in the broth at the time of plating. When these plates were further incubated there

appeared, at a later time, additional S48 colonies which were followed, in their turn, by the appearance of the S72 background growth. The later appearing S48 colonies have been regarded as representing S48 cells which arose by secondary mutations during growth of the S72 prototrophic cells on the minimal agar.

The mean number of S48 cells in the series of cultures of mutant 22A was 11.3 and the median was 11. The mean number of S48 cells in the series of cultures of mutant 34 was 17.6 and the median number was 13. From these values the rate of mutation could be determined, utilizing first, the mean value and Luria and Delbrück's (1943) expression,  $r = \alpha N_t \ln(N_t C \alpha)$ , where  $r$  is the mean number of S48 cells,  $\alpha$  the rate of mutation per bacterium per time unit,  $N_t$  the number of S72 cells at the time of plating, and  $C$  is the number of individual cultures analyzed. The rates obtained by this method were  $2.11 \times 10^{-8}$  mutations:bacterium:division cycle for mutant 22A and  $4.13 \times 10^{-8}$  mutations:bacterium:division cycle for mutant 34.

A further estimate of the rate at which S48 cells arise in these S72 prototrophic cultures was obtained from the median value,  $r_0$ , by the method of Lea and Coulson (1949). From table 3 in their paper the mean number of mutations per culture can be estimated and from this value

TABLE 2  
Generation times of various *Escherichia coli* W strains in minimal broth

Stock Culture	Technique*	Ornithine Added	No. of Determinations	Mean Generation Time†	SD
		$\mu\text{g/ml}$		<i>min</i>	
Wild type, strain W	T	0	2	62	2.8
	C	0	4	62	2.9
	T	5-15	3	60	2.5
Auxotroph 160-37	T	5-15	11	62	3.1
Pooled data on 9 F revertants from mutant 160-37	T, C	0	25	62	2.2
	T, C	10	3	63	2.5
Pooled data on 5 S48 revertants from mutant 160-37	T, C	0	20	123 → 75	4.9
	T, C	10	5	63	2.1
Pooled data on 4 S48 M* revertants from culture Z2/9	T, C	0	4	126 → 76	3.1
Pooled data on 3 S72 revertants from mutant 160-37	C	0	3	373 → 217	16.2
	C	10	1	64	
	C	10	1	66	
VS revertant from mutant 160-37‡	C	10	1	66	

\* T = automatic recording turbidimeter used; C = Coleman nephelometer used.

† Where two values are given for non-logarithmic growth, the first is an estimate of the initial rate and the second is the final rate observed. The standard deviations listed apply to the latter rates.

‡ Attempts to grow the VS strains in unsupplemented minimal broth were consistently negative.

the frequency of mutation can be derived. The frequencies obtained by this method were  $3.05 \times 10^{-8}$  mutations:bacterium:division cycle for mutant 22A and  $4.98 \times 10^{-8}$  mutations:bacterium:division cycle for mutant 34.

These two experiments, which were prompted by the high frequency with which S48 revertant types arise in S72 cultures, are the only attempts which have been made, to date, to study quantitatively the interconversion of the different prototrophic forms. Certainly, the S48 revertants are extremely stable and any attempt to perform quantitative studies similar to those described for the S72 prototrophic forms will be much more difficult.

In the plating experiments described above the prototrophic colonies were restreaked onto minimal agar for classification as soon as they appeared. Those which were subsequently classified as S48 and F appeared to be homogeneous, but in the case of those recorded as S72 the colonies frequently contained varying numbers of S48 cells which were stable in subculture. Since instability is a characteristic feature of the S72 revertants, these heterogeneous prototrophic colonies have been interpreted as originating from S72 revertant types with subsequent mutations to the S48 form during growth.

*Growth characteristics of mutant 160-37 and of the prototrophs derived from this strain.* Growth of mutant 160-37 and of representative prototrophic types in minimal broth was followed photometrically. In appropriate cases the broth was supplemented with ornithine.

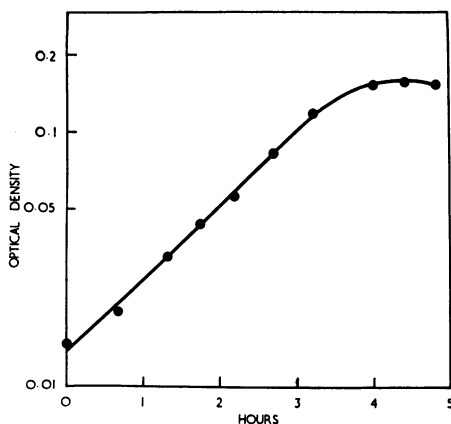


Fig. 1. Growth of *Escherichia coli* strain W (wild type) in minimal broth at 37 C. Growth was followed in the turbidimeter.

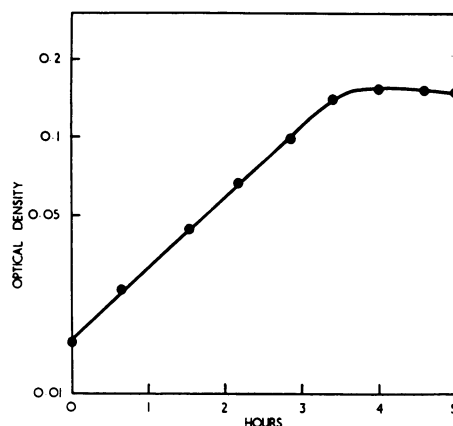


Fig. 2. Growth of *Escherichia coli* mutant 160D (F revertant) in minimal broth at 37 C. Growth was followed in the turbidimeter.

We had observed that when representatives of the slower growing prototrophs were plated on minimal agar supplemented with  $10 \mu\text{g}$  of ornithine·HCl per ml, the colonial growth was indistinguishable in size and in time of appearance from that of the wild type or of the F revertants plated on minimal agar which lacked the ornithine supplement; the generation times of the various mutants, both in minimal broth supplemented with ornithine and in minimal broth alone, are listed in Table 2.

Growth curves of the F revertants in minimal broth resembled those of the wild type (Figs. 1 and 2). The S48 revertants gave growth curves in minimal broth which were curvilinear during the logarithmic phase and exhibited increasing rates of growth (Fig. 3). During the late growth these revertants frequently achieved a rate of growth which was equivalent to a generation time of 75 min. This rate of growth approaches that of the wild type (62 min). The S72 revertants also exhibited a curvilinear growth curve (Fig. 4) and at the maximal rate of growth the generation time was 3 hr, 52 min. As a control measure to preclude a possible early mutation from the S72 state to that of an S48 and the subsequent overgrowth by the S48 cells, an appropriate dilution was made of a sample from the growth culture at the end of the experiment and 0.1 ml from this dilution was plated on minimal agar. After 48 and 72 hr of incubation, the S48 and S72 colonies were scored. In the experiment illustrated in Fig. 4, the ratio of S48 to S72 colonies was 1:300, from which we

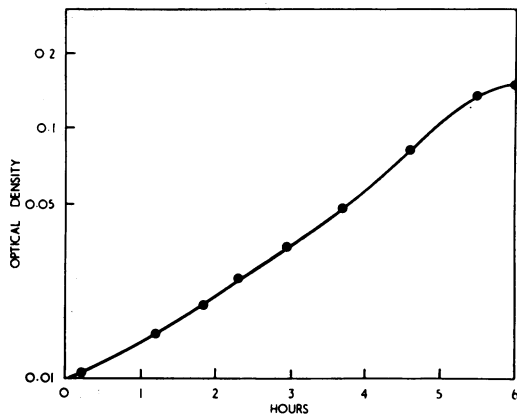


Fig. 3. Growth of *Escherichia coli* mutant 160E (S48 revertant) in minimal broth at 37 C. Growth was followed in the turbidimeter.

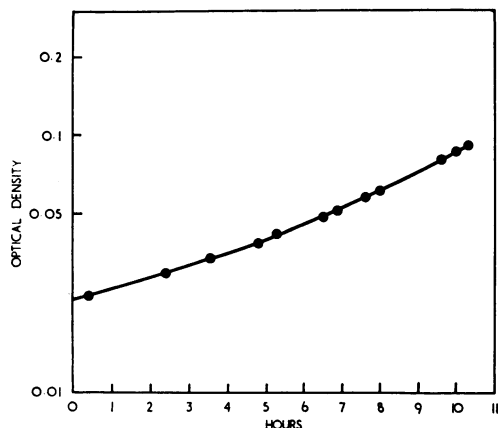


Fig. 4. Growth of *Escherichia coli* mutant 22A (S72 revertant) in minimal broth at 37 C. Growth was followed with the colorimeter.

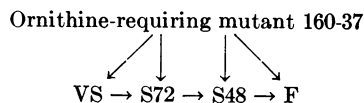
have concluded that these secondary changes did not seriously alter our quantitative results.

#### DISCUSSION

Our observations of the occurrence of slower growing or small colony type revertants are similar to those of other workers (Davis, 1950a; Bryson, 1951; Lederberg, 1952; Demerec and Cahn, 1953; Yura, 1956; and Balbinder, as cited in Demerec et al., 1958; Stadler and Yanofsky, 1959). Among the spontaneous revertants to prototrophy of the ornithine-requiring strain we observed at least four different types, ranging from a revertant (F) which appears to be indistinguishable from the wild type to a form

(VS) which grows very slowly on minimal agar and not at all in minimal broth. As is evident from the data of Table 2, in the presence of ornithine the growth rates of the S48, S72, and VS prototrophs were restored, essentially to that of the wild type and the F revertant. This observation is interpreted as indicating that in all cases the only factor limiting growth on minimal medium was a deficiency in the biosynthesis of ornithine.

Independent isolates of the three faster growing types (F, S48, and S72) appear to form homogeneous groups as judged by their growth characteristics. This grouping is also supported by data on the acetylornithinase activities of these revertants to be reported separately (Bacon and Treffers, 1961). And not only were single step isolates of a given type apparently identical, but so also were similar revertants which had arisen in a multistep fashion, for example,  $orn^- \rightarrow S72 \rightarrow S48$ . From our observations we have concluded that the following interconversions occur:



In the plating experiments, the prototrophic colonies were recorded as they appeared, singly or as "bursts" of several colonies appearing simultaneously; these latter bursts have been regarded as representing single mutational events with subsequent multiplication prior to plating. When 250 reversions were scored as to the prototrophic types, it was observed that the different forms occurred with the following frequencies: F, 20%; S48, 31%; S72, 45%; VS, 4%. This is consistent with a frequency for the spontaneous mutation from an ornithine-requiring state to that of an S48 of approximately  $0.35 \times 10^{-10}$  per bacterium, per generation. In other experiments, however, we estimated that independent S72 revertant strains gave rise to S48 forms with a frequency of about  $3.5 \times 10^{-8}$  per bacterium, per generation; this is an increase of a factor of 1,000.

If the S48 and the S72 types represent different states at a single locus, then the probability of a mutation from the S72 state to that of the S48 is much greater than that of the single step mutation from the auxotrophic state of 160-37 to that of the S48 revertant type. If, on the other hand, the S48 and the S72 types are due to events which have occurred at different loci involving, for ex-

ample, different suppressors, then it becomes necessary to postulate that when the S72 locus has been activated it serves to increase the rate of mutation at the S48 locus.

The observation that the prototrophs which arose in the presence of mutator gene were all of the S48 prototrophic type is of considerable interest. If these S48 revertants represent a particular type of mutation at the original ornithine locus then the mutator gene would appear to exhibit a high degree of specificity in the type of mutation which it induces (activates) at this locus. In the original report on the mutator system (Treffers et al., 1954), it was noted that whereas 25% of the streptomycin-resistant forms arising spontaneously in the nonmutator strain were streptomycin dependent, of those arising in the mutator strain only 0.5% were dependent forms. As it seems possible that streptomycin-resistant and -dependent forms represent mutations at a single locus (Demerec, 1950; Newcombe and Nyholm, 1950; Demerec et al., 1958), this would also be consistent with the hypothesis that mutator gene induces specific mutations at particular loci.

Alternatively, S48 revertants might also arise after the activation of a suppressor gene which is not closely linked to the ornithine locus and it is possible that mutator induces mutations only at loci linked to the mutator gene. Its influence over more distant loci (? the ornithine locus) might be indirect and depend on the presence of modifier genes within the sphere of influence of mutator.

These observations have raised a number of interesting problems which may not be adequately defined until this system can be subjected to genetic analysis. The current work of one of us (D. F. B.) is directed toward that end and already we have evidence that the mutator-induced S48 revertants represent events either at the ornithine locus itself, or at a very closely linked locus; this observation is consistent with the former hypothesis that mutator gene induces specific mutations at particular loci.

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#### SUMMARY

When spontaneous reversions to prototrophy of an ornithine-requiring mutant of *Escherichia*

*coli* strain W were examined for their growth characteristics, they were observed to be of four major types: (i) a "fast" (F) revertant which develops large colonies on minimal agar after 24 hr at 37 C, (ii) a "slow-48" (S48) revertant which requires approximately 48 hr to develop similar colonies, (iii) a "slow-72" (S72) revertant which requires approximately 72 hr, and (iv) a "very slow" (VS) form which develops small colonies on minimal agar after 5 to 7 days at 37 C. The F revertants have colonial characteristics which are indistinguishable from the wild type.

Growth of the F, S48, and S72 forms in minimal broth was followed photometrically. The F revertants were again indistinguishable from the wild type. In the case of the S48 and the S72 revertants, not only were they slower growing but their growth curves were anomalous; they exhibited increasing rates of growth during the logarithmic phase. Each of the slower growing revertants responded to ornithine.

Both the VS and the S72 prototrophic types mutated readily to faster growing forms; S72 revertants were readily obtained from the VS type and it was estimated that S72 revertants mutated to the S48 form with a frequency of  $3.5 \times 10^{-8}$  per bacterium, per generation. This rate is significantly greater than the estimated frequency of mutation directly from the auxotrophic state to that of the S48 revertant, which was only  $0.4 \times 10^{-10}$  per bacterium, per generation.

A mutator gene, which was introduced by conjugation, increased the frequency of reversion to ornithine independence 3,200-fold and all of the mutator-induced revertants which were examined were of the S48 prototrophic type. A marked specificity in mutator action is therefore evident.

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