MUTANTS OF BACILLUS SUBTILIS THAT REQUIRE THREONINE OR THREONINE PLUS METHIONINE

HOWARD J. TEAS[1]

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee[2]

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Mutant strains of microorganisms that require threonine and ones that require methionine have been reported in *Neurospora* (Horowitz, Bonner, Mitchell, Tatum, and Beadle, 1945), in *Escherichia coli* (Roepke, Libby, and Small, 1944), and in *Bacillus subtilis* (Burkholder and Giles, 1947). The finding that a monogenic *Neurospora* mutant that required both threonine and methionine could use the single amino acid homoserine (*alpha*-amino-*gamma*-hydroxybutyric acid) suggested that homoserine might be a precursor of threonine and methionine (Teas, Horowitz, and Fling, 1948). This evidence together with earlier-elucidated steps in *Neurospora* methionine synthesis (Horowitz, 1947) gives a general picture that can be verified and extended by the study of other threonine and threonine-methionine mutants.

This paper reports experiments conducted on the nutrition and mutation of *Bacillus subtilis* mutants that require threonine or threonine plus methionine for growth. The biochemical implications with respect to threonine and methionine syntheses are similar to those from *Neurospora*.

MATERIALS AND METHODS

The parental or "wild type" *Bacillus subtilis* used in these experiments was the Marburg strain, the type strain for the species (Conn, 1930), American Type Culture Collection no. 6051. For the sake of convenience this parental strain will be referred to as the normal strain. The mutant strains, kindly made available by Drs. Burkholder and Giles, were obtained by ultraviolet or by X-ray treatment of the normal strain (Burkholder and Giles, 1947). These mutants have been renumbered for convenience as follows, with the Burkholder and Giles numbers given in parentheses: 1 (146X), 2 (112X), 3 (171X), 4 (4), 5 (358), 6 (149X), 7 (0625–2), 8 (189X), 9 (187), 10 (190), 11 (186), and 12 (117X).

The minimal medium for quantitative growth experiments was that of Burkholder and Giles (1947), in which the optional asparagine was omitted and the trace element solution of Beadle and Tatum (1945) was substituted for that of Burkholder and Moyer. The formula for this double-strength minimal medium is as follows: NH_4Cl, 5.0 g; NH_4NO_3, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2$, 0.005 g;

[1] Present address: Biology Division, California Institute of Technology, Pasadena, California.

[2] Oak Ridge National Laboratory operated by Carbide and Carbon Chemicals Corporation under Contract No. W-7405-eng-26 for the Atomic Energy Commission, Oak Ridge, Tennessee.

K_2HPO_4, 3.0 g; KH_2PO_4, 1.0 g; Na_2SO_4, 1.0 g; sucrose (cp), 10.0 g; trace element solution of Beadle and Tatum, 0.25 ml; pH adjusted to 6.8; and distilled water to 500 ml.

Quantitative tests were made using 20-by-150-mm pyrex culture tubes into which 5 ml of double-strength minimal medium were pipetted; the amino acid or other supplement was added, and the volume was made up to 10 ml with distilled water. Aluminum caps or double layers of cloth toweling were used to cover the racks of tubes, which were autoclaved 12 minutes at 15 pounds pressure. The complete medium (yeast extract, tryptone, glucose agar) for keeping stock cultures was similar to that used by Burkholder and Giles (1947). The inoculum was made by taking a loopful of organisms from a 24- to 36-hour culture grown on a slant of complete medium, suspending it in 10 ml of minimal medium, and filtering this through sterile cotton. Cell counts made on such suspensions showed from 1×10^4 to 5×10^5 cells per drop. A single drop of suspension was used to inoculate each tube in all quantitative experiments.

Minimal medium for plates contained 1.5 g agar, 2.5 mg glutamic acid, and 2.5 mg arginine per 100 ml liquid minimal medium. The glutamic acid and arginine were added to the solid minimal medium since preliminary tests had shown that one or both of these were essential for colony formation by single cells.

Cultures were incubated at 30 C for 60 hours, and the density of growth was determined with a Klett-Summerson photoelectric colorimeter using a no. 54 green filter. Sixty hours was selected in order to allow maximum density without pellicle formation. Several normal strain controls on minimal medium were run in each experiment. Since the density of growth obtained for the normal strain varied somewhat from one experiment to another (usually 25 to 37 units), its density in each experiment was taken as 100 and the other densities were calculated as percentages of standard. From one experiment to another the variation in density was greater with mutants than with the normal strain. It was noted that growth densities obtained with mutants were sometimes greater than those obtained with the parental strain using similar supplements.

In reversion experiments bacterial suspensions were irradiated in lucite dishes having cellophane or thin lucite covers. The depth of the suspension was 10 to 15 mm. The radiation given was 250 KVP X-rays filtered by 3 mm aluminum. X-ray dosage was calculated from values obtained with a "victoreen" thimble ionization chamber. Doses of 90,000 to 100,000 r were used since this had been found by preliminary tests to give approximately 37 per cent survival, the point at which one would expect to obtain an average of one hit per cell and thus to recover the largest number of mutations (reversions) from a given number of original organisms (Lea, 1947). Dilutions and plating were made by standard methods; cell counts were made with a hemocytometer.

L-Cystathionine was prepared by isolation from the mycelium of methionineless *Neurospora* mutant number H98, according to the method of Horowitz (1947). Homocysteinethiolactone hydrochloride was kindly made available by Dr. N. H. Horowitz, and DL-homoserine by Dr. Marguerite Fling. Dr. R. T. Major of Merck and Company supplied the D- and L-threonine, and Dr. Marvin Armstrong supplied the D- and L-homoserine.

EXPERIMENTAL RESULTS

Nutrition of the normal strain of Bacillus subtilis. Inasmuch as the amino acid nutrition of mutants might be determined in part by that of the normal strain, the latter was tested for growth on the minimal medium and minimal medium plus each of the following 23 amino acid supplements at a level of 250 μg per tube: DL-isoleucine, DL-threonine, DL-methionine, L-histidine, DL-valine, L-lysine, L-tryptophan, L-leucine, glycine, DL-alanine, L-aspartic, L-proline, L-arginine, hydroxy-L-proline, L-glutamic, DL-serine, L-tyrosine, L-cystine, DL-norleucine, L-phenylalanine, DL-norvaline, DL-*alpha*-aminobutyric, and DL-homoserine. Of the amino acids tested, cystine brought about almost twice as much growth as

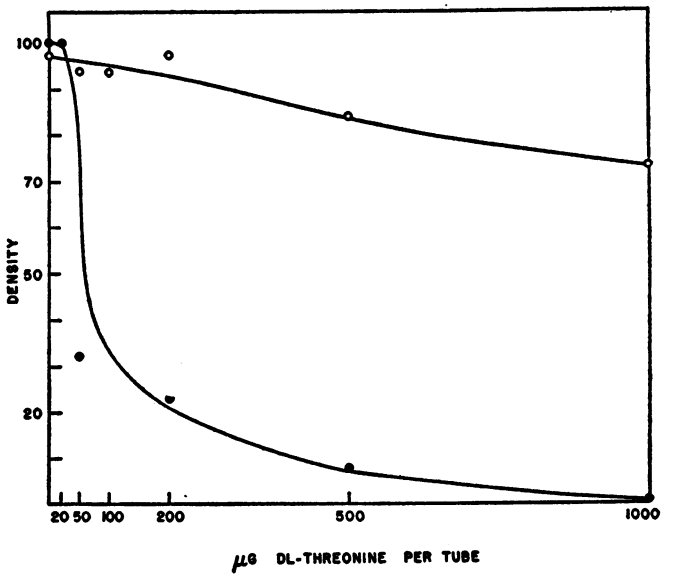


Figure 1. Growth response of the normal strain of *Bacillus subtilis* to threonine and to threonine plus glutamic acid. Dots represent growth with DL-threonine and circles growth with DL-threonine plus 250 μg L-glutamic acid per tube. Density in this and other figures and tables is given as percentage of the normal strain density on minimal medium.

minimal controls; and glutamic acid, arginine, aspartic acid, and, to a lesser extent, several others were definitely stimulatory. Threonine caused 50 to 85 per cent inhibition; norleucine, norvaline, serine, and *alpha*-aminobutyric acid caused complete inhibition. Other tests showed that glutamic acid, aspartic acid, and arginine were partially effective in overcoming the inhibitions of threonine and serine. The growth response of the normal strain to threonine and to threonine in the presence of 250 μg of glutamic acid per tube is shown in figure 1. The response of the normal strain to serine without glutamic acid, and with 250-μg and 500-μg supplements of glutamic acid per tube, is shown in figure 2.

Nutrition of mutants. None of the mutants were able to grow measurably on minimal medium in the experimental period. They were tested for ability to grow on minimal medium supplemented with any of the 23 amino acids in 250-μg

amounts. Mutants 8 to 12 were also tested on threonine plus the single amino acids. On the basis of these tests mutants 1 to 7 can be classified as threonineless; mutants 8 and 10 as homoserineless; and mutants, 9, 11, and 12 as threonine-methionineless.

The quantitative growth responses of mutants 1 to 7 to threonine were very similar; mutant 3 was taken to be respresentative. The growth response of mutant 3 to threonine and to threonine plus 250 μg glutamic acid is shown in figure 3. It can be seen that with increasing threonine growth increases to a maximum, after which it decreases as the inhibitory effect of threonine comes into play. A comparison of figure 3 with figure 1 shows that at intermediate concentrations

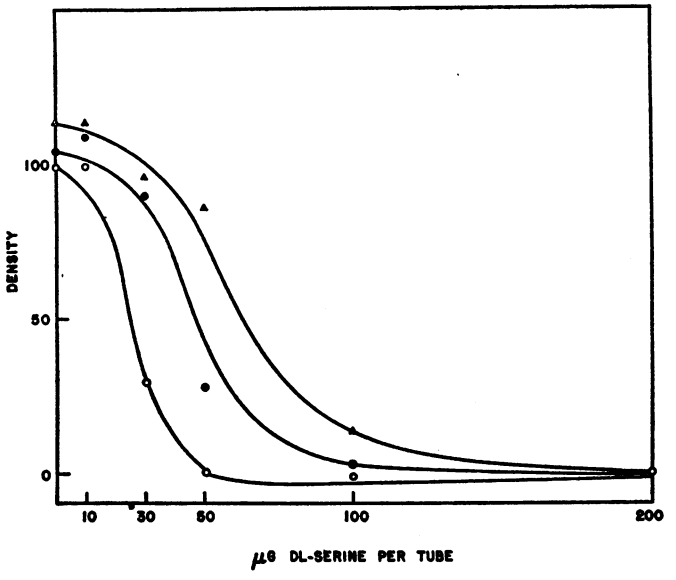


Figure 2. Growth response of the normal strain of *Bacillus subtilis* to serine and to serine plus 250-μg and 500-μg supplements of glutamic acid. Circles represent growth with DL-serine; dots growth with DL-serine plus 250 μg L-glutamic acid per tube; and triangles growth with DL-serine plus 500 μg L-glutamic acid per tube.

(100 to 200 μg per tube) the threonineless mutant was able to grow better than the normal strain. Glutamic acid overcame the inhibitory effect of threonine and gave a more "normal" curve for growth response to threonine.

The quantitative responses of mutants 8 and 10 to homoserine were similar. The growth response of mutant 10 is shown in figure 4. The growth responses of mutants 8 to 12 were tested at several levels of threonine and methionine mixtures and found to be similar. Measurable growth was initiated at 10 μg DL-threonine and 1 μg DL-methionine per tube, and was increased with larger amounts of either.

Attempts were made to substitute other amino acids for the threonine or methionine part of the growth requirements of mutants 8 to 12. For this purpose the mutants were tested on threonine plus the single amino acids for the methio-

nine replacement, and on methionine plus the single amino acids for threonine requirement. The positive results, as well as the significant negative ones, with

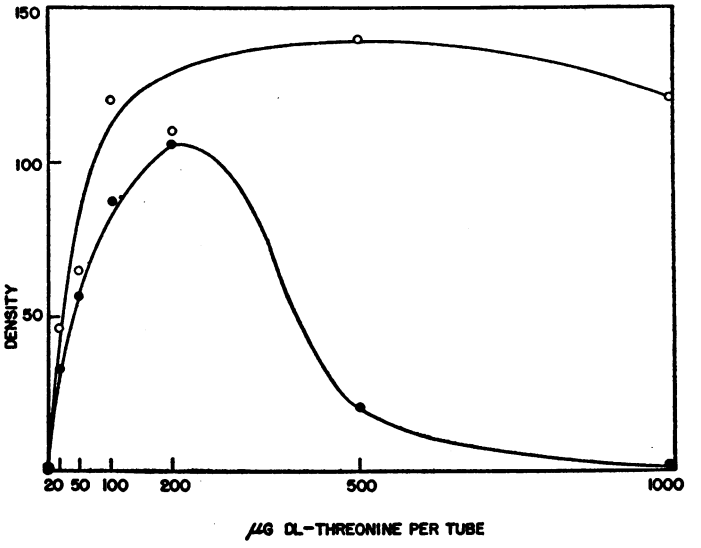


Figure 3. Growth response of threonine-requiring mutant 3 to threonine and to threonine plus glutamic acid. Dots represent growth with DL-threonine; and circles growth with DL-threonine plus 250 μg L-glutamic acid per tube.

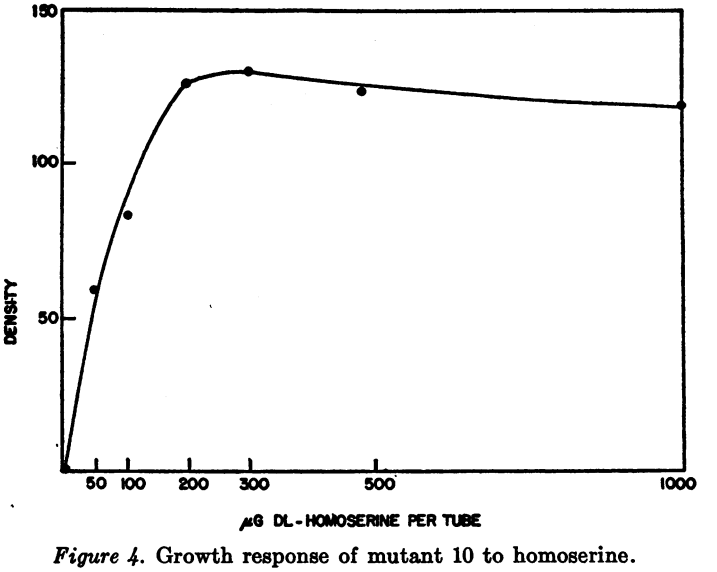


Figure 4. Growth response of mutant 10 to homoserine.

the 23 amino acids and several others known to be biosynthetically related to methionine are shown in table 1. In mutants 9, 11, and 12 methionine was replaced by homoserine. It can also be seen that cystathionine was inactive in re-

placing methionine for any of the mutants, whereas homoserine, apparently a precursor of cystathionine in *Neurospora* (Teas, Horowitz, and Fling, 1948), was active. This was at first difficult to understand; however, Binkley and du Vigneaud (1942) had found in rat liver tissue that serine plus homocysteine gave rise to cysteine, and studies of Horowitz (1947) had shown that the biosynthesis of methionine in *Neurospora* proceeds in the reverse of cysteine formation in rat liver. Therefore, it seemed possible that *Bacillus subtilis* might cleave cystathionine, presumably to give homocysteine plus serine (or something similar), and that the latter was inhibiting the growth that otherwise would come about from homocysteine. In order to check this possibility each of the mutants was retested for growth on threonine plus cystathionine with the addition of 250

TABLE 1

Quantitative growth response of mutants 8 to 12 to threonine, methionine, homocysteine, and related amino acids

AMINO ACID SUPPLEMENT* (250 μG EACH PER TUBE)	DENSITY OF GROWTH AT 60 HOURS AS PER CENT OF GROWTH OF THE NORMAL STRAIN ON MINIMAL MEDIUM				
	Mutant				
	8	9	10	11	12
DL-Homoserine	270	0	190	0	0
DL-Threonine + DL-methionine	220	176	200	248	254
DL-Threonine + DL-homoserine	233	143	200	148	191
DL-Methionine + DL-homoserine	248	0	205	0	0
DL-Threonine + DL-homocysteine†	172	214	172	248	257
DL-Threonine + L-cystathionine	0	0	0	0	0
DL-Threonine + L-cysteine‡	0	0	0	0	0
DL-Threonine + L-cystine	0	0	0	0	0
DL-Threonine + DL-homocystine	0	0	0	0	0

* Supplements of DL-threonine, DL-methionine, DL-homocysteine, L-cysteine, DL-homocystine, or L-cystathionine alone did not support growth.

† As the thiolactone hydrochloride.

‡ Cysteine was filter-sterilized.

μg of glutamic acid or 250 μg of arginine to overcome any possible serine inhibition. Tests were also made with threonine plus the approximate amounts of homocysteine and serine that would be obtained from a cleavage of 250 μg cystathionine. The results shown in table 2 indicate that the effect of threonine plus homocysteine plus serine is similar to equivalent amounts of threonine plus cystathionine in that neither would support growth. However, if glutamic acid (or in some cases arginine) is present, threonine plus homocysteine plus serine as well as threonine plus cystathionine will support growth; this suggests that the glutamic acid or arginine overcame the inhibition of serine or something associated with the conversion of cystathionine to homocysteine. The normal strain was not inhibited by cystathionine.

Experiments were conducted to determine whether only the L- or natural forms of threonine, methionine, and homoserine were utilized by the mutants.

Tests with D-, L-, and DL-threonine using mutant 3 showed that the D-isomer had no activity and the L- was approximately twice as active as the DL-mixture in the range of 50 μg to 100 μg per tube. In tests with mutant 8 it was found that D-homoserine had activity and the L-isomer was less than twice as active as DL-homoserine in the range of 50 μg to 100 μg per tube. Mutant 9 was used in testing the utilization of L- and DL-methionine. L-Methionine was more active than DL-methionine in the range of 5 μg to 20 μg per tube. With greater amounts of L- and DL-amino acids, comparisons of activity were difficult to make.

Tests were made in which a mixture of B vitamins (vitamin mixture of Beadle and Tatum, 1945) was tried for effect on the threonine, methionine, or homoserine requirements of any of the twelve mutants. The B vitamins were without

TABLE 2

A comparison of growth response of mutants 8 to 12 to threonine, cystathionine, homocysteine and serine with and without glutamic acid or arginine

AMINO ACID SUPPLEMENT, μG PER TUBE*	DENSITY OF GROWTH AT 60 HOURS AS PER CENT OF GROWTH OF THE NORMAL STRAIN ON MINIMAL MEDIUM				
	Mutant				
	8	9	10	11	12
250 DL-threonine					
+ 250 L-cystathionine	0	0	0	0	0
+ 250 L-cystathionine + 250 L-glutamic acid	172	48	167	81	133
+ 250 L-cystathionine + 250 L-arginine	10	0	0	57	147
+ 250 DL-homocysteine	172	214	172	248	257
+ 150 DL-homocysteine + 100 DL-serine	0	0	0	0	0
+ 150 DL-homocysteine + 100 DL-serine + 250 L-glutamic acid	181	191	157	200	167
+ 150 DL-homocysteine + 100 DL-serine + 250 L-arginine	195	109	167	162	186

* Supplements of threonine + glutamic acid and threonine + arginine did not support growth.

effect, indicating that none of the mutants requires threonine, methionine, or homoserine indirectly because of the inability to synthesize a B vitamin present in the mixture. Tests in which the pH of the minimal medium was altered to 5.5 and 8.0 showed no effect on the threonine, methionine, or homoserine requirements.

Genetic considerations. Syntroph formation (Lederberg, 1946) as a means of testing for allelism has been demonstrated by Lampen, Roepke, and Jones (1947), who found that certain combinations of *Escherichia coli* methionineless mutants gave increased growth over the controls. This indicated that the mutations concerned involved different genetic loci, that is, were nonallelic, since they were biosynthetically complementary types. The *Bacillus subtilis* mutants were tested for syntrophism in each of the possible combinations using large inoculations in minimal medium with and without threshold supplements of threonine

and of homoserine. The tubes were examined at the end of 3 and 5 days. No clear evidence for syntroph formation was found.

In some cases (Ryan, 1946; Giles and Lederberg, 1948) biochemical mutations are apparently reversible—that is, in a culture of mutant organisms that requires an external source of some growth factor, an individual or segment of the population occasionally appears that no longer requires the growth factor. Such reversions can be readily detected by enrichment culture procedures in which large numbers of mutant organisms are placed in an environment in which the growth factor is lacking, but which is otherwise adequate for growth. Under these conditions only the reverted organisms will grow. Ryan (1946) has found that spontaneous reversions obtained by this method occur at random with low frequency; Giles (1948) has shown that the rate of reversion of inositolless *Neurospora* mutants is increased by irradiation.

In the case of a biochemical mutant that requires two substances for growth, the requirement may be due to a single mutation (as in the case in which the synthesis of a common precursor is blocked) or it may be due to two independent mutations. One should be able to distinguish between these two possibilities by studying the occurrence and frequency of reversions on different media.

In the case of a threonine-methionineless mutant, growth of reverted and nonreverted cells would be expected to occur as follows: an unreverted mutant would not grow on minimal medium, on minimal medium plus methionine, or on minimal medium plus threonine; a mutant reverted for the threonine requirement would grow only on minimal medium plus methionine; a mutant reverted for the methionine requirement would grow only on minimal medium plus threonine; and a mutant reverted for both the threonine and methionine requirements would grow on all three media.

To determine whether a given threonine-methionineless mutant is a single or double mutant one could irradiate a suspension of the mutant strain to increase the frequency of reversion and plate out on the three media listed above. If the mutations concerned are reversible and reversions occur at random, the following results might be expected:

A. If the double requirement is due to a single mutation, the frequency of appearance of colonies on the three types of media would be approximately equal, and all colonies appearing on threonine or methionine should grow if transferred to minimal.

B. If the double requirement is due to a double mutation, the colonies appearing on methionine for the most part should represent reversions for the threonine requirement; and colonies appearing on threonine for the most part should represent reversions for the methionine requirement. Also the frequency of colonies appearing on minimal medium should be approximately the product of these two frequencies, a number that would probably be very small. In addition, most of the colonies appearing on methionine should not grow if transferred to minimal medium plus threonine or to minimal medium, and colonies appearing on threonine should not grow if transferred to minimal medium plus methionine or to minimal medium.

The results of experiments in which reversions were recovered after X-ray irradiation of mutant 8 are shown in table 3. It can be seen that the frequency with which colonies appeared on the three types of media is approximately the same (3.7, 2.0, and 2.0 $\times$ 10^{-7}). Also 29 of the colonies obtained in the two experiments (some from each of the three media in each experiment) were tested on liquid minimal medium in which all but one grew.[8] Inasmuch as the experimental results meet the conditions set forth under A and exclude those under B, it can be concluded that the double requirement of mutant 8 is due to a single mutation.

TABLE 3

Reverted colonies obtained by plating irradiated suspensions of mutant 8 on minimal medium, minimal medium plus threonine, and minimal medium plus methionine

EXPERIMENT NO.	MEDIUM					
	Minimal		Minimal plus threonine		Minimal plus methionine	
	No. colonies	Reversions per 10^7 surviving cells	No. colonies	Reversions per 10^7 surviving cells	No. colonies	Reversions per 10^7 surviving cells
1	4	5.1	5	6.4	8	10.2
2	25	3.5	11	1.5	20	1.5
Weighted average		3.7		2.0		2.0

Approximately 1 $\times$ 10^8 to 1 $\times$ 10^9 surviving cells of each of the mutants were tested by plating on the three media. Reversions were recorded only in the case of mutant 8.

DISCUSSION

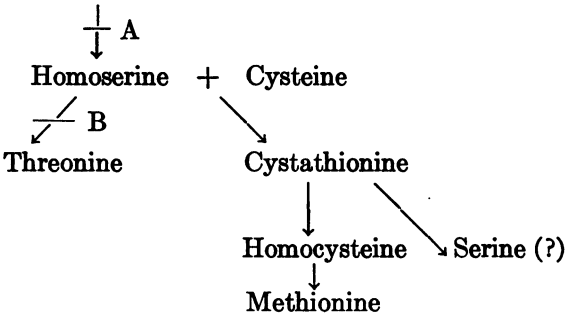
Induced biochemical mutants of fungi such as have been obtained in *Neurospora* (Beadle and Tatum, 1941; Horowitz *et al.*, 1946), *Ophiostoma* (Fries, 1943), *Penicillium* (Bonner, 1946), and *Absidia* (Giles, 1946) have in some cases been shown to differ by a single gene from the normal strain. Nutritionally similar mutants of bacteria recovered following similar radiation or chemical treatment (Gray and Tatum, 1944; Tatum, 1946; Burkholder and Giles, 1947) suggest that bacterial mutants are like the mutants of fungi in representing genically conditioned differences from the parental type.

Nutritional studies analogous to those used in establishing steps in the biosynthesis of vitamins, amino acids, purines, and pyrimidines (see reviews of Beadle, 1945, 1948) have been conducted on *Bacillus subtilis* mutants that require threonine or threonine plus methionine. From an analysis of the results

[8] Since the one colony that did not grow when transferred to liquid minimal medium came from a minimal medium plate that contained arginine and glutamic acid, it may have arisen from a cell that was reverted for the threonine and methionine requirement but which in addition had been mutated so that it required arginine or glutamic acid.

several steps in the biosynthesis of threonine and methionine can be inferred. Mutants 1 to 7, which require only threonine, must be blocked between homoserine and threonine, since of the compounds tested they utilized only threonine for growth. Mutants 8 and 10, which require homoserine (but can utilize threonine plus methionine, threonine plus homocysteine, or threonine plus cystathionine), are apparently blocked in the formation of homoserine. Mutants 9, 11, and 12, which require threonine plus methionine, can utilize homoserine to replace methionine but not to replace threonine. This suggests that there is a block in the formation of homoserine and also a block in the conversion of homoserine to threonine. Mutants 9, 11, and 12 could be called threonine-homoserineless mutants as well as threonine-methionineless mutants.

These findings can be related to the synthesis of threonine and methionine as follows:



Mutants 1 to 7 appear to be blocked at "B"; mutants 8 and 10 appear to be blocked at "A"; and mutants 9, 11, and 12 give growth responses as though they are blocked at both "A" and "B." Since attempts to distinguish among the mutants through mixed cultures analogous to heterocaryon formation in *Neurospora* (Beadle and Coonradt, 1944) were unsuccessful, there is no evidence as to whether any of the "A" blocks are the same, or whether any of the "B" blocks are the same.

The inability of *Bacillus subtilis* mutants to utilize cystathionine unless a substance was present that overcame serine inhibition suggests that serine may be produced by cystathionine cleavage. Evidence from *Neurospora* cited by Horowitz (1947) indicates that serine is not formed from cystathionine, although cystathionine is probably cleaved since it can replace methionine or homocysteine in certain methionine-requiring mutants. Inhibition of a homoserineless strain by cystathionine is not a necessary argument against cystathionine as an intermediate, since the metabolism of mutants is sometimes different from that of the normal in unpredictable ways. For instance, Doermann (1944) reported the inhibition of a lysineless *Neurospora* mutant by arginine, and Teas, Horowitz, and Fling (1948) found a homoserineless *Neurospora* mutant was inhibited by methionine. Neither arginine nor methionine is inhibitory to normal strains and both are customarily regarded as metabolic intermediates.

Reversion experiments were successful only in the type of mutant in which

the nutritional evidence for a single block was best. The failure to obtain reversions in the case of mutants 9, 10, 11, and 12 may be due to the genes concerned having low rates of reversion or having been irreversibly changed. Giles (1948) has found that differences in the rate of reversion characterize different allelic inositolless mutants in *Neurospora*. If mutants 9, 11, and 12 are blocked at both "A" and "B," then reversions for the homoserineless locus ("A" blocks) were the only type tested for, since a reversion at that locus would grow on threonine, but a reversion at the threonineless locus ("B" block) would not grow on threonine or methionine.

SUMMARY

A study of the nutrition of twelve amino-acid-requiring *Bacillus subtilis* mutants is reported. Seven required threonine; two required both threonine and methionine, but could utilize homoserine instead; and three required threonine and methionine, but were unable to grow on homoserine alone. The indications are that in *Bacillus subtilis*, as in *Neurospora*, homoserine is a precursor of both threonine and methionine.

One of the threonine-methionineless mutants was reverted to the "wild type" with a frequency of about 3×10^{-7} using 90,000 to 100,000 r of X-rays.

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