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Effects of *m*-Tyrosine on Growth and Sporulation of *Bacillus* Species[1]

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ABSTRACT

ARONSON, JOHN N. (Arizona State University, Tempe), AND GERALD R. WERMUS. Effects of *m*-tyrosine on growth and sporulation of *Bacillus* species. J. Bacteriol. **90**:38–46. 1965.—The aromatic amino acid analogue, DL-β-(3-hydroxyphenyl)alanine (*m*-tyrosine), reduced sporulation of a strain of *Bacillus subtilis* to less than 5% of that of control cultures in a glucose-salts minimal medium. The mass-doubling time increased twofold, but maximal growth equivalent to that of control cultures was eventually attained. A decreased growth rate could be maintained in the presence of the analogue for more than 10 doublings, despite incorporation of *m*-tyrosine-2-C^{14} in place of some of the protein phenylalanine. The organism proliferated to chain lengths of 10 to 15 cells. These cells persisted for many hours after maximal growth had been reached, in contrast to normal cultures which had begun to autolyze and sporulate. The response to *m*-tyrosine of strains of *B. cereus*, *B. thuringiensis*, and *B. megaterium* was like that of *B. subtilis*. In addition, *B. thuringiensis* and *B. cereus* converted *m*-tyrosine to dihydroxyphenylalanine, which was further oxidized to a melaninlike substance. Growth of a strain of *B. stearothermophilus* was not slowed by *m*-tyrosine, but a strain of *Escherichia coli* grew at a reduced rate.

The amino acid analogue, *m*-tyrosine, or β-(3-hydroxyphenyl)alanine, was reported to cause extensive chaining and to reduce sporulation of a strain of *Bacillus subtilis* (Perkins, Louie, and Aronson, 1963). Amino acid analogues are known to be capable of inhibiting bacterial sporulation (Foster and Perry, 1954), and chain formation in bacilli can be produced by antibiotics such as mitomycin C, vancomycin, D-serine (Durham, 1963), and actinomycin C (Kersten and Kersten, 1962), but the two phenomena have not been reported to be related. In general, incorporation of an amino acid analogue into bacterial protein can only occur for three or four generations before death of the organism occurs (Richmond, 1962). A more extensive investigation of the *m*-tyrosine effects produced on *B. subtilis* has revealed that this is a probable example of the incorporation of an amino acid analogue affecting the functionality of certain proteins more than others, so that total growth, although slowed, is not decreased. The normal functions of daughter-cell separation and of sporulation are drastically hindered. Some support has been obtained for the daughter-cell separation theory proposed by Webb (1951) and by Lominski, Cameron, and Wyllie (1958), who implied the necessity for a lytic factor to hydrolyze part of the outside cell wall after the new septum had been completed.

The same pattern of slowed growth and decreased sporulation was produced with *B. cereus*, *B. thuringiensis*, and *B. megaterium*, but not with *B. stearothermophilus*. A strain of *Escherichia coli* W was reported by Smith et al. (1964) to undergo 50% growth inhibition in the presence of 6×10^{-5} M L-*m*-tyrosine; in contrast, our strain of *E. coli* showed a depressed growth rate in 10^{-3} M DL-*m*-tyrosine but the same total growth as normally attained. This paper is a report of our observations regarding the sublethal effects produced by this phenylalanine antagonist on the growth and sporulation of certain species of *Bacillus*.

[1] This work was presented in part at the 144th Annual Meeting of the American Chemical Society, New York, N. Y., 1963, and at the 64th Annual Meeting of the American Society for Microbiology, Washington, D.C., 1964.

[2] Much of this work was done in the Bacteriology Department, Indiana University, and the Department of Microbiology, University of Illinois, while the senior author was a National Science Foundation Science Faculty Fellow.

[3] Part of this work was taken from a thesis submitted to the Arizona State University Graduate School in partial fulfillment of the requirements for the M.S. degree in chemistry.

MATERIALS AND METHODS

The principal organism used was a strain of *B. subtilis*, originally isolated after ultraviolet irradiation of an auxotrophic strain of *B. cereus* (Perkins et al., 1963) and erroneously considered to be a wild-type revertant. However, cesium chloride density-gradient centrifugation (Saunders, Campbell, and Postgate, 1964) of the deoxyribonucleic acid (DNA) isolated from two independent culture preparations by the procedure of Marmur (1961) revealed that the corrected buoyant density was 1.705, corresponding to a guanine-cytosine content of 41.7%. This base composition equals the reported value for *B. subtilis* and contrasts sharply with the value of 35% for *B. cereus* (Lee, Wahl, and Barbu, 1956). Our organism was undoubtedly isolated as a contaminant, rather than a prototrophic revertant. The variant used for this study was resistant to methionine sulfone (Aronson, 1963).

The strain of *B. megaterium* was obtained from E. D. Weinberg, the strain of *B. cereus* T was obtained from the laboratory of H. Orin Halvorson, *B. thuringiensis* was originally obtained from T. A. Angus, and *B. stearothermophilus* 1503-4R was obtained from L. L. Campbell. *E. coli* ML30 and ML48 were obtained from H. V. Rickenberg.

B. subtilis was grown either in the salts-0.15% glucose medium described previously (Perkins et al., 1963) or in a modification of medium E of Vogel and Bonner (*unpublished data*). A 50-fold concentrate for the latter medium was made as follows: to 67 ml of deionized water were added: $MgSO_4 \cdot 7H_2O$, 1 g; citric acid·$1H_2O$, 10 g; K_2HPO_4, 50 g; $NaNH_4HPO_4 \cdot 4H_2O$, 17.5 g; and $CHCl_3$ (as preservative), 0.2 ml. A 2-ml amount of the concentrate plus 0.4 ml of a salt solution containing 0.5 mg each of $FeCl_3 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$ was diluted to 100 ml with deionized water and autoclaved. Sterile glucose (0.4 ml of 50%) was added aseptically to give a final concentration of 0.2%. *B. thuringiensis* and *B. cereus* were grown in the above medium supplemented further with 1 ml each per 100 ml of medium of 1% solutions of L-methionine and L-glutamic acid, or, alternatively in the case of *B. cereus*, with the six amino acids as suggested by Nakata (1964), except that the L-glutamic acid was only 18.4 mg per 100 ml of medium. *B. megaterium* was grown in the synthetic medium described by Weinberg (1964) or in the modified Vogel-Bonner medium described above for *B. subtilis*. *B. stearothermophilus* was grown in the minimal medium of Welker and Campbell (1963) supplemented with 14 amino acids equivalent to 0.1% casein hydrolysate minus phenylalanine. *E. coli* was grown in either the mineral medium of Cohn and Monod (1951) with 0.15% glucose or in the modified Vogel-Bonner medium described above for *B. subtilis*. All inhibitor concentrations were 10^{-3} M of the DL isomer, and the inhibition was the same for the alternate media mentioned above.

Growth and sporulation experiments were usually carried out with 25 ml of medium in 125-ml side-arm flasks; growth was measured by absorbance at 600 mμ in a Bausch & Lomb Spectronic-20 colorimeter, and could be related to standard curves for dry weight or protein. Incubation was at 37 C on a rotary shaker or, in the case of *B. stearothermophilus*, at 55 C in a water-bath shaker. Colonies produced by plating on nutrient agar after prior heating at 70 C for 15 min were considered to be derived from spores rather than from vegetative cells. Morphological changes of vegetative cells were followed by microscopy of crystal violet-stained smears or by viewing suspensions of viable cells in a Petroff-Hausser chamber.

Ribonucleic acid (RNA), DNA, and protein determinations were made with cell powders prepared by washing harvested cells from synchronized cultures twice with 5% trichloroacetic acid and then extracting the cells in succession with cold 95% ethyl alcohol, cold ethyl alcohol-chloroform (3:1), cold ethyl alcohol-ether (3:1), and, finally, acetone at −20 C. The residual powders were digested in 1 N KOH at 33 C for 18 hr, and samples of this digest were taken for protein and RNA determinations. Protein was determined by the modified biuret procedure of Habermann (1961) with egg albumin (Nutritional Biochemicals Corp., Cleveland, Ohio; our analysis, 82.8% protein versus crystalline urease; all samples corrected accordingly) as a standard. RNA was determined by the *p*-bromophenylhydrazine procedure of Webb (1956) with reagent-grade RNA (Nutritional Biochemicals Corp.) as a standard. DNA was determined in a separate portion of the cell powder by a slight modification of the ultraviolet absorption procedure of Buetow and Levedahl (1962) with A-grade DNA (Calbiochem) as standard.

The DL-*m*-tyrosine used was obtained from Sigma Chemical Co., St. Louis, Mo., or from A. G. Fluka Co., Buch, Switzerland, and proved to be chemically (by chromatography and infrared spectra) and microbiologically identical to a batch subsequently synthesized according to the procedure of Sealock, Speeter, and Schweet (1951). DL-*m*-Tyrosine-2-C^{14} was prepared in the same manner, starting with glycine-2-C^{14} obtained from Volk Radiochemical Co., Burbank, Calif. (lot no. 2740, N, specific activity of 5.0 mc/mmole). A 1-mc amount (15 mg) was admixed with 4.0 g of carrier glycine, dissolved in 16 ml of water, and acetylated by the addition of 12 ml of acetic anhydride (Blatt, 1947). Two recrystallizations from hot water yielded 5.48 g of *N*-acetylglycine-2-C^{14} (87.8% yield). The entire *m*-tyrosine-2-C^{14} synthesis was carried out with a radiochemical yield of 11.7%. The labeled *m*-tyrosine was recrystallized from hot water to constant specific activity. Purity was checked further by thin-layer chromatography with the use of isopropyl alcohol–2 N HCl (65:35, v/v) as a solvent. Only one spot appeared (R_F of 0.75) upon radioautography and after

spraying with ninhydrin. Radiochemical determinations were made with the aid of a Nuclear-Chicago gas-flow counter. Radioautograms were made with Kodak Medical X-ray film.

Protein was isolated from cells by the procedure of McQuillen and Roberts (1954). The crude protein residue was further extracted with 1 N NH_4OH; fractions of this extract were dried directly on planchets for radiochemical determinations. The extract was lyophilized, and 5-mg fractions were hydrolyzed with 1 ml of 6 N HCl in sealed tubes for 24 hr at 110 C. The hydrolysates were evaporated to dryness over NaOH in an evacuated desiccator and taken up in water for thin-layer chromatography or in *p*H 2.2 citrate buffer for amino acid analysis on a Spinco amino acid analyzer by the procedure of Spackman, Stein, and Moore (1958).

RESULTS

B. subtilis showed a typical bacterial growth pattern in which the initial inoculum of 2×10^6 to 3×10^6 cells per milliliter increased to a final cell density of 8×10^7 to 10×10^7 cells per milliliter after 6 to 7 hr (Fig. 1). The doubling time was about 55 min during the log phase. In contrast, cells grown in the presence of DL-*m*-tyrosine (5×10^{-4} to 1×10^{-3} M) exhibited slowed growth, with the time for doubling the cell mass being lengthened to 90 to 100 min (Fig. 1). Maximal growth was about equal to that of the untreated cultures, but was not reached until several hours afterwards. The control cultures consisted of motile diads, triads, and single cells; only occasionally were longer chains observed, and this was usually during the late lag or early log phase. In contrast, the *m*-tyrosine cultures appeared nonmotile and consisted almost entirely of very long chains (10 to 15 cells) when viewed by phase microscopy or in crystal violet-stained smears. Cross walls were seen distinctly, and the cells appeared slightly elongated and more narrow than the control cells. The chains were found to persist and lengthen throughout the growth cycle; they usually remained intact up to 40 hr after maximal growth, whereas control cultures normally began lysing as a prelude to sporulation as soon as the stationary phase was reached. By 18 to 24 hr, the control cultures contained 6×10^7 to 7×10^7 spores per milliliter; in contrast, *m*-tyrosine cultures had less than 5%, and often less than 1%, of this number of spores at 24 to 48 hr. The inhibitory effect of *m*-tyrosine on the rate of growth and the extent of sporulation was negated by the addition of either 200 mg per liter of enzyme-hydrolyzed purified casein (Nutritional Biochemicals Corp.) or 5×10^{-4} M L-phenylalanine. To reverse the inhibitory effect of the analogue, the phenylalanine had to be added early during the penultimate division (A = 0.20 to 0.25; see Fig. 1). Conversely, to decrease the growth rate and to inhibit sporulation, the analogue had to be added by A = 0.20.

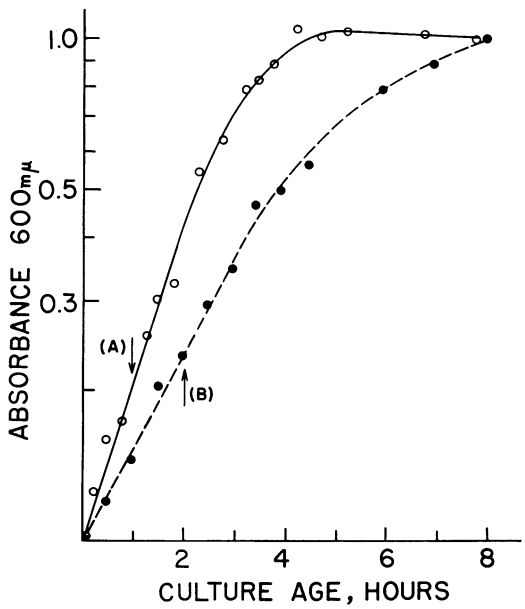

FIG. 1. *Growth inhibition of Bacillus subtilis by 10^{-3} M DL-m-tyrosine. Symbols: ○ = normal growth; ● = inhibited growth. (A) Last period for m-tyrosine addition to produce inhibition of a growing culture in a glucose-salts medium. (B) Last period for phenylalanine addition to reverse m-tyrosine inhibition.*

The analogue-inhibited cultures were shown to be capable of normal growth rates and normal sporulation by suspending them in fresh medium containing no analogue. Cells thus treated and incubated in mineral medium without glucose for 1 hr to allow for the efflux of *m*-tyrosine resumed normal growth without any lag upon the addition of glucose. Even in the presence of the analogue, the cells could be transferred numerous times, with growth always being at the reduced rate until a resistant mutant was selected. Routine experiments involved at least 4 doublings in the analogue-medium, and sometimes 10.

A gross analysis of cell powders from the inhibited cultures and normal cultures was undertaken to determine whether the *m*-tyrosine cells had an abnormal composition (Table 1). Biuret analysis (Habermann, 1961) for the total protein content of the control culture cell powders showed a slight increase during logarithmic growth, followed by a decrease in the early stationary phase when some lysis had begun. The weight-per cent protein values were 51% at

early log phase and 58% at the maximal growth level; they then dropped to 53% after 2 hr in the stationary phase. The *m*-tyrosine-inhibited cells contained overall lower amounts of protein and a correspondingly higher proportion of an unidentified alkali-insoluble material. This latter substance was found to be ninhydrin-positive, contained negligible phosphorous, and was suspected to be cell-wall material. Since cell-wall synthesis is known to be independent of protein biosynthesis (Shockman, 1963), one might expect that cultures grown in the presence of an analogue that slows down protein synthesis might have a higher weight-per cent of cell-wall material. The *m*-tyrosine cell preparations showed no consistent trend in protein levels with culture age; the values ranged from 45 to 50%.

The control samples showed a variation in RNA content from 18.6 weight-per cent at early log phase, 14.6 at the maximal growth level, and back up to 16.3% after 2 hr in the stationary phase. The *m*-tyrosine cell powders showed very little variation in RNA level, the values being 13.8 to 14.5%, significantly lower than those of control cultures.

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The DNA values for the control samples decreased from 2.2% during early log phase to 1.7% at maximal growth. The *m*-tyrosine cell preparations had only 1.0 to 1.9% DNA, perhaps due to the slower rate of growth which would be consistent with the decrease in DNA content of *B. cereus* from 2.2 to 1.1% in the presence of 5-fluorouracil, as reported by Reich and Mandel (1964).

The normal protein–RNA–DNA ratio for *B. subtilis* was reported by Spotts and Szulmajster (1962) to be about 18:9:1, which is approximately what we obtained. In the case of the cell powders from control cultures, 71 to 74% could be accounted for as protein, RNA, and DNA. This total is in agreement with the reported value of 26% of dry weight for the cell-wall material of *B. cereus* (Kronish, Mohan, and Schwartz, 1964). The analogue-inhibited culture had only 61 to 66% protein plus nucleic acid, another indication of an increased percentage of cell-wall material.

Several organisms that could be grown in a synthetic medium were examined to learn whether the effects of *m*-tyrosine upon the growth and sporulation of *B. subtilis* were also extant for other species of *Bacillus*. A strain of *E. coli* was also included in this study to determine whether the analogue affected only *Bacillus* species. The results are summarized in Table 2 and Fig. 2. *B. stearothermophilus* was the only organism upon which the analogue exerted no deleterious effect, perhaps because the synthetic medium used was a very rich one, although devoid of phenylalanine. Sporulation of *B. cereus* and *B. thuringiensis*, and also parasporal "crystal" formation in the case of the latter organism, was drastically reduced, whereas sporulation of *B. megaterium*, despite a slowed growth rate, was delayed only 12 hr. The maximal turbidity of the inhibited cultures in all cases eventually equaled (within 10%) that of the control cultures. The slightly decreased viable-cell counts for inhibited cultures are an indication of the extent of chain formation, most evident in the case of *B. subtilis* which did not form chains in the control medium. In all cases, early addition of 5×10^{-4} M L-phenylalanine completely reversed the effects of the inhibitor.

B. cereus and *B. thuringiensis* *m*-tyrosine cultures began to darken near maximal growth and had become black after 24 hr. The supernatant fraction of a *B. thuringiensis* culture just beginning to darken gave a pink color by the procedure of Arnow (1937), indicative of dihydroxyphenylalanine. *m*-Tyrosine itself gives a yellow color with this test. Presumably *B. cereus*

TABLE 1. *Protein, RNA, and DNA content of Bacillus subtilis*

Substance	Control cell powders					*m*-Tyrosine cell powders				
	3.2 (0.33)*	4.0 (0.43)	5.0 (0.64)	6.5 (0.85)	(08.5 .75)	5.8 (0.33)	6.9 (0.45)	7.8 (0.57)	10.0(0.)80	11.50.78)
Protein...	511	536	547	578	530	478	485	448	497	506
RNA.....	186	160	158	146	163	144	140	138	138	145
DNA.....	22	22	18	17	17	10	13	19	16	12
Total...	719	718	723	741	710	632	638	605	651	663

* Culture age (hours), with absorbance at 600 mμ in parentheses. All values are micrograms per milligram of cell powder.

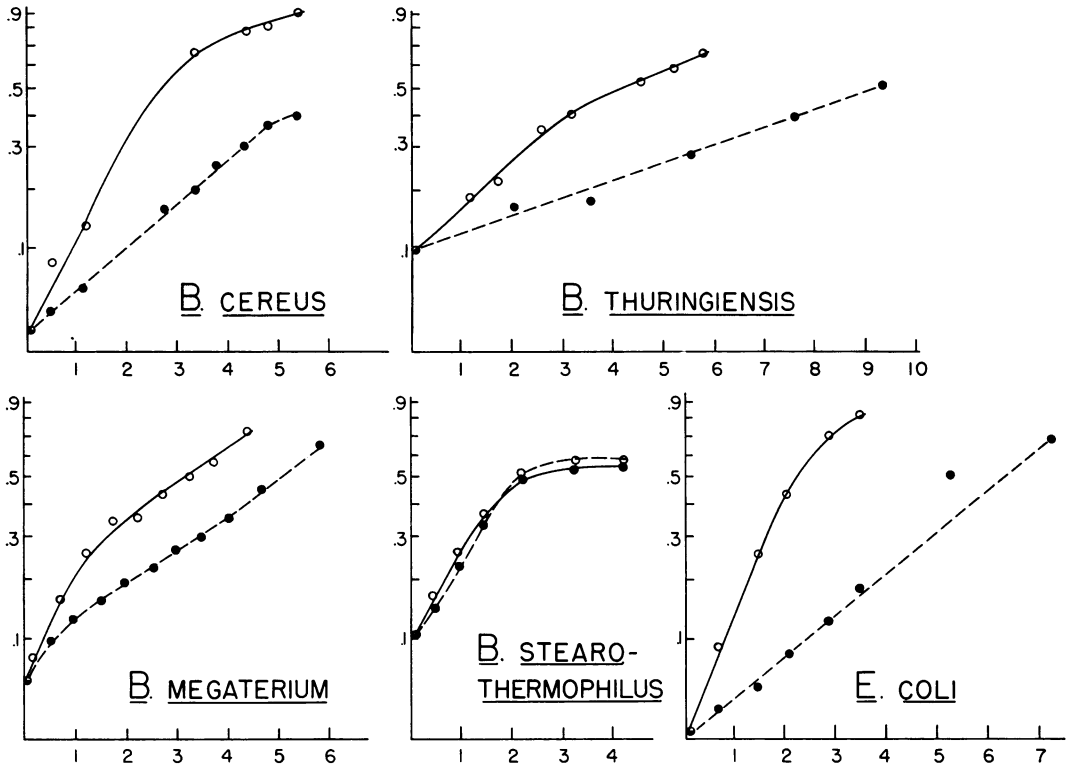

FIG. 2. *Effect of* DL-*m-tyrosine on growth rate of several microorganisms. Ordinates: logarithmic plot culture absorbance at 600 mμ. Abscissas: growth time in hours. Solid lines = normal medium. Broken lines = normal medium supplemented with* 10^{-3} M *inhibitor.*

and *B. thuringiensis* contain an oxidase that converts *m*-tyrosine (but not tyrosine) to dihydroxyphenylalanine, which then undergoes autocatalytic oxidation to "melanin." The "melanin" tenaciously adhered to cellular debris and was insoluble in water, buffer, chloroform, and dimethylformamide. Neither the dihydroxyphenylalanine nor the "melanin" seemed to affect the growth and sporulation of *B. thuringiensis*, since 10^{-3} M DL-dihydroxyphenylalanine allowed the normal doubling time of 120 to 130 min and normal sporulation, despite the presence of the rapidly forming "melanin." The other organisms caused negligible production of "melanin," even after 4 to 6 days on the shaker. At 10^{-3} M, dihydroxyphenylalanine caused a twofold increase in the doubling time of *E. coli*. The viable colony count at 24 hr was only 1.7×10^9 per milliliter compared with the control value of 4.3×10^9 per milliliter.

The slowed growth of the inhibited organisms is typical of situations in which an amino acid analogue is being incorporated into protein (Richmond, 1962). Two approaches were used to verify that *B. subtilis* was incorporating this analogue: the organism was grown in the presence of *m*-tyrosine-2-C^{14}, and amino acid analysis of protein from *m*-tyrosine-grown cells was performed with an automatic amino acid analyzer. The protein purified by the procedure of McQuillen and Roberts (1954) and solubilized in 1.0 N NH_4OH contained 1.2×10^3 disintegrations per min (dpm) per mg. From the specific activity (1.8×10^5 dpm/mg) of the *m*-tyrosine-2-C^{14}, and with the use of a residue weight of 163 (181 − 18 to allow for water removed in peptide bond formation), it is estimated that the protein contained 4.0 μmoles of *m*-tyrosine per 100 mg of protein. Radioautography of a fraction of an acid hydrolysate of the C^{14}-protein revealed only a single radioactive spot corresponding to *m*-tyrosine. Amino acid analysis of the hydrolysate of a nonradioactive inhibited-protein sample revealed a small *m*-tyrosine peak emerging two-thirds of the distance from the leucine to the tyrosine peak, but completely resolved from the tyrosine peak. A value of 0.6% was obtained (Table 3) when the *m*-tyrosine was expressed as percentage of the

TABLE 2. *Effect of m-tyrosine on growth rate and sporulation of several organisms**

Culture	Organism	Mass doubling time	Colony-formers per ml	Spores per ml
		min		
Control	*Bacillus subtilis*	55	7.4×10^7	6.7×10^7
	B. megaterium	60	6.0×10^7	5.8×10^7
	B. thuringiensis	130	7.5×10^7	7.1×10^7
	B. cereus T	60	3.0×10^8	4.1×10^7
	B. stearothermophilus	75	—	—
	Escherichia coli ML 30	50	4.3×10^9	—
With DL-*m*-tyrosine, 10^{-3} M	*B. subtilis*	90	3.0×10^7	4.6×10^5
	B. megaterium	130	1.8×10^7	2.6×10^7†
	B. thuringiensis	330	9.6×10^6	3.9×10^4
	B. cereus T	135	2.5×10^7	5.0×10^5
	B. stearothermophilus	75	—	—
	E. coli ML 30	130	3.9×10^9	—

* Viable and spore determinations were made on 30-hr cultures; spore determinations were repeated at 48 hr.

† Sporulation was delayed 12 hr and later reached the same value as control cultures.

eleven amino acids most stable to the hydrolysis procedure, according to Sueoka (1961). The combined *m*-tyrosine plus phenylalanine (5.6%) closely approximated the percentage of phenylalanine (5.8%) in noninhibited protein. Since there was a relative increase, rather than a decrease, in tyrosine in the inhibited protein, it seems likely that the *m*-tyrosine was indeed primarily replacing phenylalanine, and not tyrosine, in the protein. Since the "stable" amino acids only account for about 75% of the total amino acids, the amount of *m*-tyrosine found in the protein by amino acid analysis would correspond to 2.8 μmoles per 100 mg. This value is somewhat lower than that obtained by measuring the incorporation of C^{14}-analogue, although not greatly so. It does seem evident that approximately 10% of the phenylalanine of normal protein was replaced by the analogue.

After numerous transfers of *B. subtilis* through medium containing *m*-tyrosine, stained smears revealed that an increasing proportion of the cells were no longer growing as long chains; eventually the growth rate was equal to that of the control cultures. Mutants resistant to amino acid analogues are well known (Cohen and Adelberg, 1958; Fangman and Neidhardt, 1964; Lewis, 1963; Ramakrishnan and Adelberg, 1964). The *m*-tyrosine-resistant mutant shows genetic derepression of the natural synthetic pathway with concomitant overproduction of the natural amino acid or its precursor; when the resistant mutant was streaked on nutrient agar on either side of a streak of *E. coli* ML 48 (a phenylalanine leaky mutant that will grow readily with either phenylalanine or phenylpyruvic acid), the *E. coli* grew out in 12 hr. The *E. coli* auxotroph would not grow when streaked between dual streaks of the *m*-tyrosine-sensitive strain of *B. subtilis*. The resistant strain sporulates normally in the presence of *m*-tyrosine, and maintains its resistance after being subcultured on nutrient agar.

TABLE 3. *Amino acid composition of Bacillus subtilis total protein**

Amino acid	Control culture	*m*-Tyrosine culture
Lysine	11.5	9.8
Histidine	2.9	3.2
Arginine	5.7	7.1
Aspartic X	14.1	14.0
Glutamic X	20.5	20.9
Proline	4.2	4.0
Alanine	10.1	9.3
Valine	10.2	9.8
Leucine	10.5	11.2
Tyrosine	4.5	5.1
Phenylalanine	5.8	5.0
m-Tyrosine	—	0.6
(Phenylalanine + *m*-tyrosine)	—	(5.6)
Total	100.0	100.0

* Values are percentages of 11 stable amino acids (Sueoka, 1961).

Biosynthesis of a new cell septum subsequent to nuclear division and prior to cell separation occurs centripetally towards the longitudinal axis of the cell (Chapman and Hillier, 1953). The separation process would seem to require prior digestion of the residual outer cell wall that binds the two daughter cells together (Chung,

Hawirko, and Isaac, 1964). Experiments were undertaken to ascertain whether the inhibition of lysis of *B. subtilis* and the formation of chains produced by *m*-tyrosine were facets of the same phenomenon, that is, the decreased production of an active lytic factor as a result of the analogue incorporation into cell protein.

The inherent ability of *m*-tyrosine-grown cells to be lysed by normal culture supernatant fluids was first verified by suspending the chained cells in the basal medium without glucose. Samples (1 ml) of this suspension were added to 2 ml of a normal 24-hr culture supernatant liquid and to a 2-ml portion that had been previously boiled for 5 min. In 24 hr at 37 C, the absorbance at 600 mμ dropped from 1.5 to 0.35, whereas the control (boiled) remained at 1.5. The turbidity at 600 mμ of a wall preparation from normal *B. subtilis* cells decreased from $A = 0.52$ to $A = 0.25$ when incubated for 45 min with the supernatant fluid from a 24-hr normal culture, and decreased from 0.51 to 0.28 when incubated with the same supernatant fluid diluted 1:1 with that of a *m*-tyrosine culture; however, the turbidity only decreased from 0.56 to 0.45 when incubated with the supernatant fluid from a 26-hr *m*-tyrosine culture. Two observations can be readily made: the normal supernatant liquid had more lytic activity than did a *m*-tyrosine supernatant fraction, and the latter did not contain an inhibitor of the normal lytic factor.

A suspension of *m*-tyrosine-cells was centrifuged and suspended in basal medium without glucose. Samples were then mixed 1:1 with appropriate supernatant fractions and incubated overnight with shaking at 30 C. The chain suspensions were then examined in a Petroff-Hausser chamber under phase microscopy. The unheated culture supernatant fraction from an uninhibited culture reduced the average number of cells per chain from 10 to 2, with a concomitant increase in number of single cells from <25 to $>40\%$. A separate experiment was performed in which *m*-tyrosine chains were aseptically centrifuged and suspended in the modified Vogel-Bonner salts solution. Volumes of 1 ml were diluted with an equal volume of either sterile water or Millipore-filtered, staled, normal culture supernatant liquid. After incubation for 45 min at 37 C, appropriate dilutions were made with sterile water, and samples were plated on nutrient agar. Incubation in normal supernatant fluid increased the number of colony-formers twofold, from 7.6×10^7 to 16.5×10^7 per milliliter.

DISCUSSION

The physiological abnormalities of *B. subtilis* described above are of interest because replacement with *m*-tyrosine of approximately 10% of the phenylalanine component of the cell protein altered two seemingly unrelated phenomena, cell separation and sporulation; yet, total growth and viability remained unimpaired. Others (Chung et al., 1964; Lominski et al., 1958; Webb, 1951) have shown the necessity for enzymatic fission of part of the pre-existing wall for cell separation, and our work indicates that this enzymatic process is affected by the altered protein produced with the analogue. The proportion of the cell-wall material was increased in the inhibited cultures, but the walls were still susceptible to lysis by the normal enzyme.

Although the sporulating cell is still capable of utilizing exogenous amino acids (and presumably amino acid analogues, as well) at least until the stage of spore inclusion within the cell (Millet and Aubert, 1960), there was no indication that *m*-tyrosine interfered with sporulation when added to the culture very late. It seems more likely that the *m*-tyrosine-grown cells could not achieve the RNA and protein readjustment required for "commitment to sporulation" (Foster and Perry, 1954). A question that remains to be answered is to what extent required metabolic reshuffling is dependent upon growth rate alone. The slowed growth rate in *m*-tyrosine cultures could be due to factors other than just incorporation of the analogue into protein. Smith et al. (1964) showed that *m*-tyrosine exerted an in vitro inhibition of the *E. coli* phenylalanine-sensitive 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) synthetase and a repression of the tyrosine-sensitive DAHP synthetase. Previc and Binkley (1964) showed that another phenylalanine antagonist, *p*-fluorophenylalanine, also interfered with both the phenylalanine and the tyrosine metabolism of *E. coli*. However, added tyrosine only partially enhanced the growth rate of our inhibited *B. subtilis*, whereas phenylalanine completely restored the culture to normal conditions. This could be explained by either a sparing mechanism for joint phenylalanine-tyrosine precursors or a mechanism by which phenylalanine can be directly converted to tyrosine.

The partial replacement of phenylalanine by *m*-tyrosine did not adversely affect the majority of the cell proteins. This assumption is reasonable in light of the demonstration by Janeček and Rickenberg (1964) that β-galactosidase had unchanged catalytic activity despite a majority of the phenylalanine residues being replaced by β-2-thienylalanine. Orgel (1964) had discussed a mechanism by which physiological adaptation to the incorporation of an analogue can readily occur when many enzymes are slightly affected,

but not completely inactivated. Such a situation may also be in operation with *B. subtilis* in *m*-tyrosine. The effect of *m*-tyrosine on the growth and sporulation of other *Bacillus* species was similar. The oxidation of *m*-tyrosine to dihydroxyphenylalanine has previously been reported in the rat (Sourkes, Murphy, and Rabinovitch, 1961); it is of interest to note that, of the organisms we tested, only *B. cereus* and *B. thuringiensis* carried out this conversion; yet, they did not do so with the natural tyrosine.

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