JOURNAL OF BACTERIOLOGY, Oct. 1970, p. 351-359
Vol. 104, No. 1

Metabolic Influences on Tyrosine Excretion in *Bacillus subtilis*[1]

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Received for publication 24 February 1970

The biosynthetic pathway for tyrosine synthesis is regulated both by repression of enzyme synthesis and by feedback inhibition of enzyme activity in *Bacillus subtilis*. Nevertheless, wild-type cells produce significantly more tyrosine than is required for protein synthesis, and part of this is excreted into the medium. Alteration of nutritional and other environmental conditions of cultivation strongly influenced the amount of tyrosine excretion. The excretion of tyrosine by wild-type cells was compared with that of a regulatory mutant having a feedback-insensitive prephenate dehydrogenase. Tyrosine excretion varied directly with the in vitro activity of prephenate dehydrogenase and inversely with temperature in the two strains. The regulatory mutant excreted five times as much tyrosine as the wild type at all growth temperatures examined. The carbon source used for growth significantly influenced the level of tyrosine excretion. The specific activity of prephenate dehydrogenase was also affected by the carbon source. Incorporation studies with isotopic tyrosine and fluorometric determinations of tyrosine concentrations extractable in hot water were used to measure operationally the tyrosine pools in the mutant and wild-type strains. The effects of various environmental conditions on the synthesis and excretion of tyrosine led to the conclusion that metabolic controls governing end product contrations exist which are completely independent of regulation by feedback inhibition and repression.

The appearance of free amino acids in culture supernatant fluids of growing bacterial cells has been described for a number of different microorganisms (10). This appears to be inconsistent with the efficient control of biochemical pathways that is generally thought to characterize bacterial cells. The loss of such compounds from growing cells has been related to protein turnover (18) and to fermentation processes (8, 16). The production of extracellular glutamic acid by a *Bacillus* species has been reported (5); tyrosine excretion by wild-type *B. subtilis* reported in this communication is another example of this phenomenon.

The efficient disposition of endogenously synthesized metabolites by microorganisms requires rigorous control over their production and utilization. End product-mediated inhibition of enzyme activity and repression of enzyme synthesis are usually considered to be the major constraints on the amount of product made by a biosynthetic pathway. Ordinarily, these regulatory mechanisms are sufficient to prevent excretion of the end product into the medium. Loss of either control by mutation may constitute a severe selective disadvantage. The possible effects of different environmental conditions upon the completeness of the regulation exercised by repression and feedback inhibition have generally not been studied.

To investigate the effect of altered growth conditions on amino acid production, we have compared L-tyrosine excretion in a regulatory mutant of *B. subtilis* with that of a wild-type strain. The mutant, which lacks feedback control of prephenate dehydrogenase by tyrosine, is resistant to the growth-inhibitory effects of the D-isomer of tyrosine (4). We have examined the influence of temperature changes and various carbon sources upon the tyrosine excretion in these strains. The quantitative production of tyrosine in vivo was correlated with the activity of prephenate dehydrogenase in vitro and with the internal pool size of L-tyrosine.

MATERIALS AND METHODS

Strains. All experiments described were conducted with *B. subtilis* NP 40, a prototrophic derivative of

[1] Presented in part at the 69th Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 4-9 May 1969.

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strain 168 *trpC* (3), and with NP 164, a D-tyrosine-resistant mutant which excretes L-tyrosine. The isolation and characterization of NP 164 has been described (4).

Growth media. Minimal salts solution consisted of the following in grams/liter: K_2HPO_4, 14; KH_2PO_4, 6; disodium citrate, 4; $MgSO_4$, 0.2; and $(NH_4)_2SO_4$, 2. Trace elements were added as before (12). Sterile glucose at a final concentration of 0.5% was added after autoclaving. For experiments employing single carbon sources, minimal salts without citrate were supplemented with the alternative carbon compound at 0.5% after separate sterilization.

Chemicals. Radioactive ^{14}C-L-tyrosine (microliters) at 395 mCi/mM was purchased from New England Nuclear Corp., Boston, Mass. Barium prephenate was isolated and purified as previously described (4). Amino acids, cofactors, and compounds used as carbon sources were the products of Calbiochem, Los Angeles, Calif., or Sigma Chemical Co., St. Louis, Mo., and were of the highest quality available. Other chemicals were of reagent quality.

Growth conditions. Growth was monitored by measuring the turbidity at 600 nm in a DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Sample volumes of 2 ml were taken at appropriate times from 200-ml shake cultures grown in 1-liter flasks. For temperature studies, cells were grown in a New Brunswick Psychrotherm shaker, at 350 rev/min, with the temperature controlled at ±0.05 C. All inocula for temperature experiments were portions of a culture grown in minimal medium at 37 C which were stored frozen in liquid nitrogen. Experiments were begun by adding 2 ml of thawed inoculum to prewarmed media. For experiments testing different carbon sources, overnight cultures were grown on 0.5% glucose in minimal salts lacking citrate and were diluted 50-fold in the new growth medium to initiate the experiment. These cultures were grown on a New Brunswick platform shaker at 200 rev/min at 37 C. Growth is expressed in terms of the specific growth rate k (hr^{-1}) (9).

The relation of optical density at 600 nm to cell dry weight was determined by trapping exactly 5 ml of a suspension of washed cells of known turbidity on preweighed membrane filters (0.45 μm pore size; Millipore Corp., Bedford, Mass.), and then drying the filters to constant weight. An optical density of 1.0 at 600 nm is equivalent to 0.45 mg (dry weight) of cells/ml.

Tyrosine excretion. To analyze tyrosine present in culture supernatant fluids, 2-ml samples were removed at intervals from growing cultures, the optical density was measured at 600 nm and the cells were removed by filtration through a membrane filter (25 mm diameter, 0.45 μm pore size; Millipore). The fluorescence of 1 ml of the filtrate was measured in an Aminco-Bowman Spectrophotofluorometer, using an excitation wavelength of 275 nm and an emission wavelength of 325 nm (uncorrected). The temperature was maintained thermostatically at 37 C. A standard curve was used to relate tyrosine concentration to fluorescence intensity.

The qualitative identification of tyrosine in culture supernatant fluid was verified by paper chromatography of concentrated supernatant fractions. Tyrosine was identified by its reaction with ninhydrin and with diazo-*p*-nitroanaline after 7 hr of descending chromatography in butanol-acetic acid-water (4:1:5) on Whatman no. 4 paper. Other fluorescent aromatic phenols were not detected. In addition, concentrated supernatant material from NP 164 contained sufficient tyrosine to support a local growth response of a tyrosine auxotroph of *B. subtilis* on minimal medium plates spotted with 0.05 ml of supernatant fluid.

Tyrosine pool: analysis by isotopic uptake. Tyrosine pools were measured by the following techniques. Ten ml of a culture grown in minimal medium (37 C) to an optical density of 0.30 to 0.50 was placed in a 50-ml beaker. The sample was stirred vigorously on a combination hot plate-magnetic stirrer at about 37 C. After 10 min of preincubation, 0.1 ml of ^{14}C-L-tyrosine (diluted 40-fold with carrier ^{12}C-L-tyrosine) was added to give a final concentration of 10^{-6} M (0.01 μCi/ml). At appropriate intervals, 0.2-ml samples were removed. One half of each sample was filtered through a membrane filter and washed with 3.0 ml of prewarmed growth medium containing 100 μg of L-tyrosine per ml. The remainder of the sample was added to 0.9 ml of 10% trichloroacetic acid containing 2.0 mg of L-tyrosine per ml and held on ice for 30 min. These latter samples were then filtered through membrane filters and washed with 10 ml of 5% trichloroacetic acid containing 1 mg of L-tyrosine per ml. All samples were air dried under an infrared lamp, placed in scintillation vials containing 10 ml of toluene-based counting fluor (Omnifluor, New England Nuclear Corp.), and counted in a CPM scintillation spectrophotometer (Beckman Instruments, Inc.) at greater than 75% efficiency. This procedure is similar to that described by Kay and Gronlund (14).

Tyrosine pool: hot water-extractable tyrosine. At intervals during growth on glucose minimal medium, 25-ml samples were removed from 200-ml cultures; the absorbancy at 600 nm was measured, and the cells were pelleted by centrifugation at 10,000 × *g* for 15 min. The supernatant fluid was retained to assay for exogenous tyrosine and the cells were washed with fresh growth medium. The washed cells were resuspended in 3 ml of distilled water and the endogenous tyrosine was released by boiling the suspension for 30 min. Cell debris was removed by centrifugation and the supernatant fraction was assayed fluorometrically for tyrosine.

Enzymology. Cells grown to the end of the exponential phase of growth were pelleted by centrifugation at 3000 × *g* for 10 min and resuspended in 3.0 ml of 0.05 M potassium phosphate buffer (*p*H 7.8) containing 0.1 M KCl. Whole cells were often stored frozen at −20 C prior to the preparation of extracts. Extracts for enzyme analysis were made as described previously (4). Barium prephenate was greater than 95% pure. All assays for prephenate dehydrogenase activity were conducted in the Aminco-Bowman spectrophotofluorometer, with the substrates barium prephenate and nicotinic adenine dinucleotide at 1.0 and 1.2 mM, respectively. Other assay conditions have been described (4). A circulating water bath was used to maintain constant temperatures in the cuvette chamber. Enzyme specific activity is expressed as nanomoles of

reduced nicotinic adenine dinucleotide (NADH) formed per minute per milligram of protein. Protein determinations were made by the procedure of Lowry et al. (17) with bovine serum albumin as a standard.

RESULTS

Temperature effects on tyrosine excretion. The growth rate of *B. subtilis* NP 40 in glucose minimal medium at various incubation temperatures is indicated in Fig. 1. The logarithm of the specific growth rate, plotted against the reciprocal of the absolute temperature, in an Arrhenius plot, defines a normal growth range (11) between 32 and 42 C. NP 164, in this range, had a diminished rate of growth due to its partial requirement for phenylalanine (4).

The linear increase in tyrosine present in culture supernatant fluids as a function of increasing cell mass (calculated from optical density changes) is compared for each strain in Fig. 2. Since both quantities increase in an exponential fashion with time, a straight line results when one is plotted against the other. With increasing temperature, a progressive decrease in the amount of excreted tyrosine was observed. The excretion of tyrosine in the mutant NP 164 paralleled that in NP 40, but was five- to sixfold greater at each temperature. This fact is best seen in Fig. 3, in which the tyrosine excreted is given as a function of the reciprocal of the absolute growth temperature.

The difference in growth rates of the two strains at each temperature complicates a comparison of the amount of tyrosine excreted per unit time. This difficulty was eliminated by multiplying the absolute excretion at any temperature by the specific growth rate at that temperature. This new value, defined as the specific tyrosine excretion

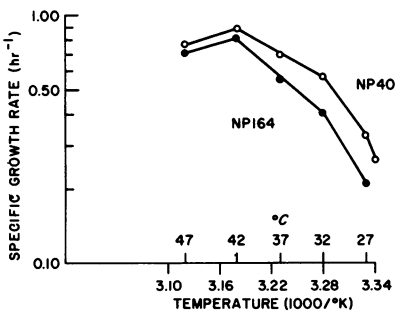

FIG. 1. *Specific growth rate of strains NP 40 and NP 164 as a function of reciprocal of growth temperature. Symbols:* ○, *specific growth rate of NP 40 at temperatures indicated;* ●, *specific growth rate of NP 164. Ordinate: logarithm of specific growth rate (hours⁻¹). Abscissa: reciprocal of growth temperature (degrees Kelvin).*

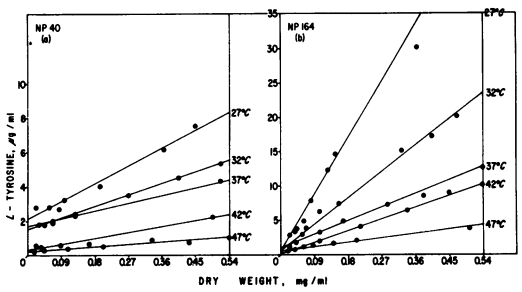

FIG. 2. *Tyrosine excretion in NP 40 and NP 164 as a function of growth temperature. (a) Tyrosine excretion by NP 40 at temperatures indicated. (b) Tyrosine excretion by NP 164 at temperatures indicated. Ordinate: L-tyrosine in culture supernatant fluids (µg/ml). Notice different ordinate scales in a and b. Abscissa: cell dry weight (mg/ml).*

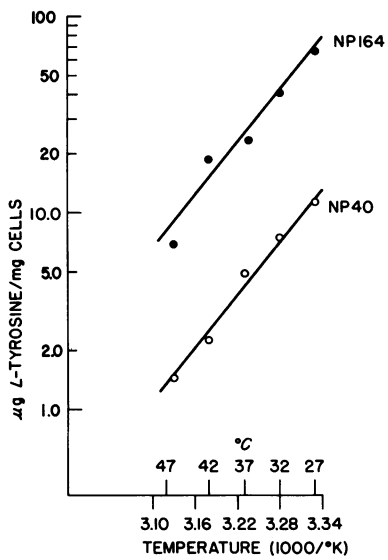

FIG. 3. *Tyrosine excretion in NP 40 and NP 164 as a function of reciprocal of growth temperature. Symbols:* ○, *µg of tyrosine excreted per mg of cells in NP 40 at growth temperature indicated;* ●, *µg of tyrosine excreted per mg of cells in NP 164. Ordinate: logarithm of tyrosine excreted (µg/mg of cells). Abscissa: reciprocal of growth temperature (degrees Kelvin).*

(E_{tyr}), expresses the micrograms of tyrosine excreted per milligram (dry weight) of cells per hour. Explicitly, if T is the concentration of tyrosine, x is the cell mass, and t is time, then $E_{tyr} = (1/x)\,(dT/dt)$ and $E_{tyr} = (1/x)\,dT/dx)\,(dx/dt)$. Since $(1/x)\,(dx/dt) = k$ (reference 9), then $E_{tyr} = k\,(dT/dx)$, where dT/dx is the slope (at each temperature) from Fig. 2. This quantity is plotted against the reciprocal of the absolute

growth temperature for each strain in the upper part of Fig. 4. This formulation, in contrast to that in Fig. 3, includes a rate term, and therefore is in proper form for an Arrhenius plot.

Prephenate dehydrogenase is the first enzyme in the aromatic pathway which is specific for the biosynthesis of tyrosine (20). The lower half of Fig. 4 is an Arrhenius plot of the activity of prephenate dehydrogenase assayed in vitro at the temperatures indicated. The similarity of the shapes of the upper and lower curves suggests that the temperature dependence of the specific tyrosine excretion of each strain reflects the in vivo activity of prephenate dehydrogenase. The specific activity of the enzyme was not significantly influenced by the growth temperature or the growth phase of the cells at the time of harvest.

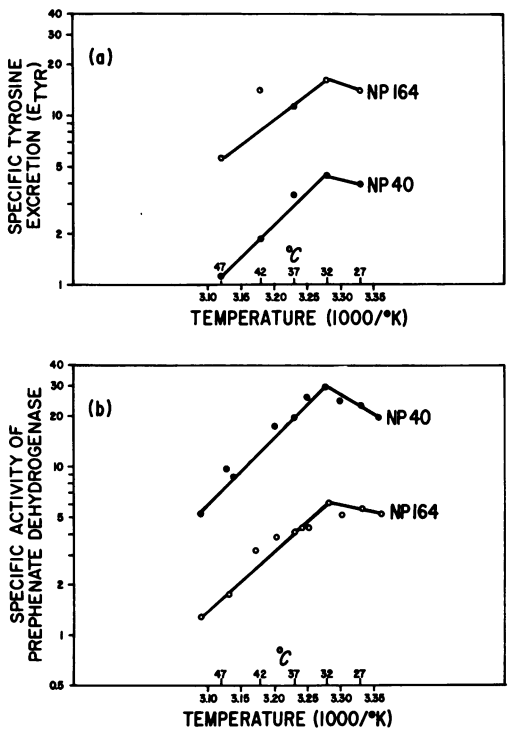

FIG. 4. *Specific tyrosine excretion and specific activity of prephenate dehydrogenase as a function of reciprocal of temperature. (a) Ordinate: logarithm of specific tyrosine excretion (μg of tyrosine per mg of cells per hr) for NP 40 (●) and NP 164 (○). Abscissa: reciprocal of growth temperature (degrees Kelvin). (b) Ordinate: logarithm of specific activity of prephenate dehydrogenase (nmoles of NADH per min per mg of protein) for NP 40 (●) and NP 164 (○). Abscissa: reciprocal of assay temperature (degrees Kelvin). Enzyme was assayed in crude extracts of cells grown at 37 C. Reaction mixture contained 0.156 mg of protein from NP 40 and 0.475 mg of protein from NP 164.*

The activity of prephenate dehydrogenase isolated from NP 164 showed the same temperature profile as did the enzyme from NP 40 (Fig. 4b). The specific activity in NP 164 was reduced fourfold, however, by the repressive effects of the tyrosine overproduced by this mutant (4).

Effect of different carbon sources upon tyrosine excretion. Figure 5 shows that the source of carbon during growth strongly influences the relative amounts of tyrosine excreted by NP 40 and NP 164. A characteristic excretion pattern in the presence of each carbon source was observed. As noted in the temperature studies, the variation of excretion in NP 40 paralleled that of NP 164. In the temperature studies, in which both glucose and citrate were present as sources of carbon, NP 164 excreted five times as much tyrosine as did NP 40. When only single sources of carbon were present, the differential was diminished to a value of 2 to 3 (ratios at the top of Fig. 6a). L-Tyrosine itself cannot serve as a source of carbon for *B. subtilis* (*unpublished data*). The specific tyrosine excretion obtained in the presence of various carbon sources for both strains is indicated in Fig. 6a. The absence of citrate from the glucose growth medium (used in the temperature studies) diminished the amount of tyrosine excreted by about twofold for NP 40 but caused a fourfold decrement in the amount excreted by NP 164.

The relative specific activities of prephenate dehydrogenase in extracts from cells cultured on the various carbon sources are compared in Fig. 6b. The repressed enzyme levels in NP 164 are expressed by the ratios at the top of this figure. The three variable parameters (specific growth rate, specific enzyme activity, and specific tyrosine excretion) measured after growth on various carbon sources are summarized in Table 1. Neither the specific excretion of tyrosine nor the specific activity of prephenate dehydrogenase was necessarily proportional to the specific growth rate obtained during growth on a given carbon source. Citrate yielded specific tyrosine excretion values equal to or better than those obtained after growth on glucose. This was accomplished with about half as much enzyme activity, a reasonable result considering that intracellular concentrations of tyrosine are diluted only half as fast by growth. Although pyruvate and ribose support similar rates of growth, ribose apparently is a much better source of early precursors of the aromatic amino acids (erythrose-4-P and P-enolpyruvate). The elevated specific activity of prephenate dehydrogenase during growth on pyruvate probably reflects derepression in response to low end product levels of tyrosine.

Tyrosine pool: isotopic uptake. The incorpora-

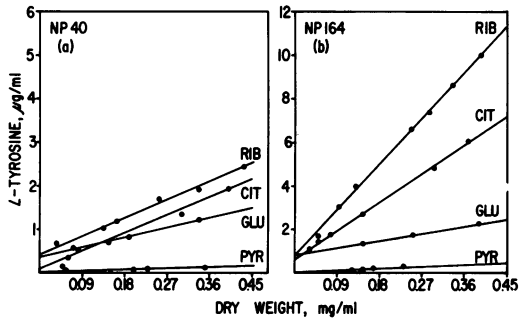

FIG. 5. *Effect of carbon source upon tyrosine excretion in NP 40 and NP 164. (a) Tyrosine excretion by NP 40 growing on carbon sources listed. (b) Tyrosine excretion by NP 164 growing on carbon sources listed. Ordinate: L-tyrosine in culture supernatants (μg/ml). Notice different ordinate scales in a and b. Abscissa: cell dry weight (mg/ml). Abbreviations: GLU, glucose; CIT, citrate; RIB, ribose; PYR, pyruvate.*

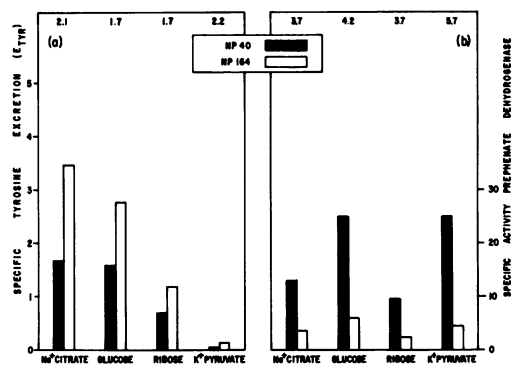

FIG. 6. *Influence of carbon source upon specific tyrosine excretion and specific activity of prephenate dehydrogenase in NP 40 and NP 164. (a) Specific tyrosine excretion of NP 40 and NP 164 growing on carbon sources indicated. Ratios of specific excretion of NP 164 to NP 40 given at top. Ordinate: specific tyrosine excretion (μg of tyrosine per mg of cells per hr). Abscissa: carbon source used. (b) Specific activity of prephenate dehydrogenase of NP 40 and NP 164 grown on carbon sources indicated. Ratios of specific activities of NP 40 to NP 164 given at top. Ordinate: nmoles of NADH per min per mg of protein. Abscissa: carbon source used.*

tion of radioactive tyrosine into whole cells and into protein in each of the two strains is compared in Fig. 7. Tyrosine in this pool is operationally defined as the difference between these two quantities (2, 7). The relative rates of uptake of tyrosine into the cell were 0.40 and 0.03 μmoles per min per g of cells (dry weight) for NP 40 and NP 164, respectively (Table 2). A calculation of the maximum amount of isotopic tyrosine in the pool (from Fig. 7) showed that 0.3 μmoles of tyrosine/g of cells filled the pool of NP 40 (Table 2). For NP 164 the amount was 0.05 μmoles/g. Since six times as much isotopic tyrosine per unit weight was taken up by NP 40, the tyrosine pool of NP 164 presumably is nearly saturated with endogenously synthesized tyrosine. Because NP 164 excretes appreciable amounts of tyrosine, its rate of uptake may reflect the degree of exchange between endogenous and exogenous tyrosine. For both strains a ratio of 1:15 for exogenous tyrosine taken up into the pool (at maximum) to total tyrosine incorporated into protein was observed. In 30 min, all of the added tyrosine was removed from the medium by NP 40.

TABLE 1. *Effect of carbon source on specific tyrosine excretion, specific growth rate, and specific activity of prephenate dehydrogenase*

Carbon source	NP 40			NP 164		
	E_{tyr}[a]	k[b]	Specific activity[c]	E_{tyr}	k	Specific activity
Citrate....	1.66	0.26	13.0	3.45	0.23	3.5
Glucose...	1.60	0.59	25.0	2.76	0.46	6.0
Ribose....	0.70	0.14	8.5	1.17	0.05	2.3
Pyruvate..	0.06	0.16	25.0	0.12	0.12	4.4

[a] Expressed as micrograms of tyrosine per milligram of cells per hour.

[b] Expressed as hours^{-1}.

[c] Expressed as nanomoles of NADH per minute per milligram of protein.

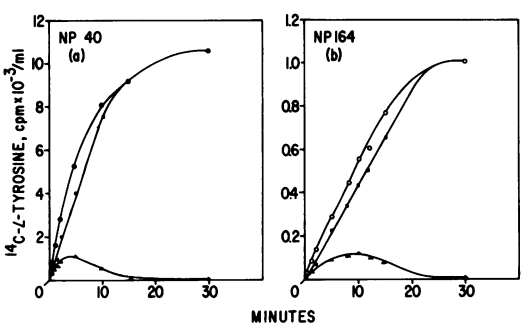

FIG. 7. *^{14}C-L-tyrosine incorporation in NP 40 and NP 164. (a) ^{14}C-L-tyrosine incorporated into whole cells (●) and proteins (■) of NP 40. Pool tyrosine calculated as difference (▲). (b) ^{14}C-L-tyrosine incorporated into whole cells (○) and proteins (□) of NP 164. Pool tyrosine calculated as difference (△). Ordinate: ^{14}C-L-tyrosine incorporated, counts/min × 10^{-3} per ml of cell suspension. Note different ordinate scales in a and b. Abscissa: time in minutes. NP 40 contained 0.25 mg of cells per ml; NP 164 contained 0.14 mg of cells per ml. L-Tyrosine ^{12}C + ^{14}C added to give 10^{-6} M final concentration (0.01 μCi/ml).*

Figure 7 reveals that pool tyrosine became labeled simultaneously with the uptake of label into protein. This rapid, linear uptake of tyrosine into protein without a lag implicates passage through a small pool with a high turnover.

Tyrosine pool: hot water-extractable tyrosine. Endogenously synthesized tyrosine extracted from cells growing exponentially in minimal medium without added tyrosine was a constant fraction of the dry weight. For NP 40 growing in minimal medium, 2.0 μmoles of tyrosine/g (dry weight) was measured in the endogenous tyrosine pool. In NP 164, there were 5 μmoles/g. These amounts are listed in Table 2 along with the calculations for constants derived from the analysis of the expandable pool in these strains.

DISCUSSION

The excretion of tyrosine by wild-type *B. subtilis* suggests that control over tyrosine biosynthesis may be incomplete in this microorganism. It would appear that the ability of the primary feedback controls to regulate tyrosine production may be tempered by other metabolic influences. Some tyrosine excretion may result from protein turnover or cell lysis, or both. Protein turnover, with the concomitant release of amino acids into the extracellular environment, has been shown to occur in growing cultures of *B. subtilis* (22) and *B. cereus* (24). However, calculations based on pool sizes and the rates of protein synthesis indicate that these processes cannot account for more than a small fraction of the tyrosine observed in culture supernatant fluids. For example, in NP 40 grown on glucose at 37 C, 14 times as much tyrosine is excreted as resides in the endogenous pool.

The exact reasons for the excretion by wild-type cells are not known; this loss of tyrosine contrasts with growing cells of *Escherichia coli* B in which, under similar conditions, no tyrosine can be detected in culture supernatant fluid (*unpublished data*). Studies on the regulation of prephenate dehydratase, the first enzyme specific for the biosynthesis of phenylalanine in *B. subtilis*, suggest that the excretion of tyrosine may be an important aspect of a regulatory interaction between tryptophan and phenylalanine synthesis (23). Tyrosine excretion may also be related to the dominating role of this amino acid on the repression control of the enzymes for aromatic amino acid biosynthesis in *B. subtilis* (21).

The enzymological basis of the tyrosine overproduction of NP 164 was the insensitivity of prephenate dehydrogenase to feedback inhibition by tyrosine (4). A consequence of this lack of metabolic control on tyrosine biosynthesis was a compensating fourfold repression of prephenate dehydrogenase synthesis in this mutant. This strain grew more slowly on minimal medium than its parent, NP 40. Phenylalanine supplementation restored the wild-type growth rate in NP 164.

In both strains, temperature affected the growth rate in a fashion similar to that described for other microorganisms (11). The optimal growth temperature was 42 C. Strain NP 164 had an increased temperature characteristic over the normal growth range compared to NP 40. This temperature effect was presumably a reflection of its altered requirement for phenylalanine for optimal growth at higher temperatures. The decreased tyrosine excretion in this mutant at elevated growth temperatures should serve to alleviate the drain of phenylalanine precursors into tyrosine synthesis and thus reduce the phenylalanine limitation, resulting in an enhanced growth rate.

Since the amount of tyrosine excreted at any given time by a growing culture is a function both of the number of cells present and of their rate of growth, it is important that both variables be considered in analyzing tyrosine excretion. For example, Fig. 3 indicates that the "apparent" tyrosine excretion was greatest at 27 C. When the slower growth rate at this temperature is also considered, the specific excretion of tyrosine per milligram of cells per hour was less than that

TABLE 2. *Characteristics of tyrosine pools*

Strain	^{14}C-L-tyrosine uptake[a]	Saturation at 10^{-6} M	Tyrosine pools[b]	
			Isotopic uptake	Hot water-extractable
NP 40	0.40	−	0.30	2.0
NP 164	0.03	+	0.05	5.0

[a] Expressed as micromoles per minute per gram. Calculations for strain NP 40 are as follows: 3×10^3 counts/min per ml per 2 min per 0.25 mg of cells per ml ✠ = 6×10^3 counts/min per min per mg = 4×10^{-3} μCi per min per mg★ = 10.2×10^{-6} μmoles per min per mg (^{14}C-L-tyrosine) ℂ = 4×10^{-4} μmoles per min per mg (total tyrosine) ✳ = 0.4 μmole per min per g. In these calculations, the uptake was assumed to be linear for the first 2 min; the cell densities are given in the legend to Fig. 7. Symbols: ✠, cells of NP 40 at A_{600} of 0.55 = 0.25 mg of cells per ml (Fig. 7); ★, 1.6×10^6 counts/min per μCi of ^{14}C-L-tyrosine (75% efficiency); ℂ, 395 μCi per μmole (specific activity); ✳, 1:40 dilution of ^{14}C-L-tyrosine to ^{12}C-L-tyrosine.

[b] Expressed as micromoles per gram. Calculations were performed as described above for maximum level of isotopic tyrosine found in the expandable pool, i.e., at 5 min for NP 40 and at 10 min for NP 164 (Fig. 7).

at 32 C (Fig. 4). The apparent tyrosine excretion of each strain is given as a function of temperature of growth in Fig. 3 and illustrates two facts. The first is that the magnitude of the excretion by NP 164 is about fivefold greater than that of NP 40. Second, the slopes of the two lines are equal, suggesting that other cellular controls, independent of control by feedback inhibition or repression, influence the total synthesis of tyrosine.

When the specific tyrosine excretion was examined as a function of growth temperature, 32 C was optimum in both strains. The curves defining the specific excretion in NP 40 and NP 164 differ somewhat because of the different effects of temperature on the growth of each. If the strains grew at identical rates at each temperature (or if the ratio of their rates were constant over the temperature range examined), the curves for the specific excretion would have the same shape, since the lines in Fig. 3 are parallel. Thus, the relative specific excretion of the two strains is normalized when the different temperature effects on growth are considered.

The loss of tyrosine into the growth medium by growing cells correlated with the in vitro specific activity of prephenate dehydrogenase when both were followed as a function of temperature. For both strains, the optimal temperature for excretion and enzyme activity was 32 C, and the quantitative effects of temperature on excretion and enzyme activity parallel one another. This suggests that temperature largely affects tyrosine excretion in correspondence with its effect upon the in vivo activity of the enzyme.

With the temperature constant, the growth rate depended upon the available source of carbon. The growth rates on different carbon compounds observed here compare favorably with those described by others for *B. subtilis* (6, 19). Tyrosine excretion in these strains was distinctly influenced by the carbon source utilized for growth. Presumably, the specific tyrosine excretion has a high value on carbon sources which generate specific precursors for aromatic amino acids well, relative to ability to support growth in general.

Varied carbon compounds undoubtedly affect tyrosine excretion by altering the rates of production and amounts of intermediary metabolites available for the biosynthesis of this amino acid. In general, Table 1 indicates that a diminished specific growth rate is correlated with a decreased enzyme specific activity, reflecting the general decline in total carbon metabolism. Citrate could support growth at only half the rate characteristic of glucose, and the enzyme specific activity declined accordingly. For carbon sources such as pyruvate and ribose which support approximately the same growth rate, the specific excretion was inversely correlated with the specific activity of prephenate dehydrogenase. This probably reflects the varying ability of different carbon sources to supply precursors to the specific pathway for aromatic amino acid biosynthesis. Since tyrosine has been shown to be an effective repressor of prephenate dehydrogenase and of other enzymes for aromatic biosynthesis (21), alterations in the amounts of precursors available for aromatic biosynthesis should affect the amounts (specific activities) of enzymes in the aromatic pathway, and thus the amount of tyrosine made and excreted. For a given carbon source, the specific tyrosine excretion is therefore determined both by the growth rate and by the enzyme specific activity characteristic of that carbon compound.

These conclusions are analogous to those reached by Jensen and Neidhardt (13) concerning the effect of growth rate on the extent of histidine catabolism and histidase synthesis in *Aerobacter aerogenes*. They demonstrated that a partial coupling exists between histidine catabolism and growth rate. Intracellular levels of histidase were also affected by the growth rate. In this analysis of an anabolic pathway, similar conclusions seem to apply concerning the amounts of tyrosine made (and excreted) as a function of the growth rate and enzyme levels.

The analysis of tyrosine pools in these two strains suggests that tyrosine excreted by the mutant is used to fill an expandable pool, which is immediately accessible to protein synthesis. The diminished rate and extent of incorporation of isotopic tyrosine in NP 164 reflects its dilution and competition for uptake by the endogenously synthesized tyrosine which is excreted. For each strain, a ratio of 1:15 was found for exogenous tyrosine in the expandable pool to total isotopic tyrosine incorporated. This shows that exogenous tyrosine, once taken up, is equally accessible to protein synthesis in both NP 40 and NP 164. The influence of temperature or altered carbon source on the pool size of either strain is not known; differences in tyrosine excretion under certain conditions may be related to differences in the loss of tyrosine from this pool.

The distinction between an expandable pool for exogenous amino acids and an internal pool for endogenous amino acids in *Candida utilis* has been clearly stated by Cowie and McClure (7). We have no evidence for such functionally distinct pools in *B. subtilis*. Hot water-extractable tyrosine was 2.5 times greater in NP 164 than in NP 40. Small pools and the rapid incorporation of exogenous amino acids into protein may be

a characteristic of *Bacillus* species, since a similar observation has been made by Bernlohr (1) with respect to the uptake and incorporation of amino acids in *B. licheniformis*.

To relate the demand for tyrosine in protein synthesis with the relative amounts excreted, calculations utilizing the data describing growth and tyrosine excretion at different temperatures (Fig. 1 and 4) are presented in Table 3. For NP 40, the calculations indicate that the amount of tyrosine used (for protein synthesis) or lost (via excretion) varies two- to threefold. However, the ratio of the amounts of tyrosine entering protein to tyrosine excreted varies almost ninefold since the two change oppositely with increased temperature. Thus at 27 C, about 50% of the tyrosine molecules are used for growth. At 47 C, by contrast, almost 90% of the tyrosine synthesized is used to support growth.

In NP 164, on the other hand, at low temperatures almost 90% of the tyrosine made is lost by excretion. Only at temperatures greater than 42 C does tyrosine used for growth exceed that excreted. These calculations suggest flexible controls over the synthesis and utilization of tyrosine in NP 40; in NP 164 the loss of feedback inhibition results in an excess of tyrosine at all but the highest temperatures examined.

TABLE 3. *Protein synthesis and tyrosine excretion in NP 40 and NP 164 at different growth temperatures*

Strain	Growth temp	k^a	Synthesis[b]	Protein excretion[c]	Ratio of synthesis to excretion
	C				
NP 40	27	0.33	4.25	3.68	1.15
	32	0.57	7.25	4.30	1.68
	37	0.69	8.80	3.36	2.62
	42	0.83	10.60	1.84	5.75
	47	0.76	9.70	1.09	8.90
NP 164	27	0.21	2.68	14.0	0.19
	32	0.40	5.10	16.0	0.32
	37	0.52	6.65	11.2	0.59
	42	0.76	9.70	14.1	0.69
	47	0.70	8.95	5.6	1.60

[a] Expressed as hours^{-1} (9).

[b] Expressed as micrograms of tyrosine per milligram of cells per hour. Calculations are based on protein as 51% of cell dry weight and tyrosine as 2.5% of protein (*unpublished data*). Tyrosine for protein synthesis equals 12.7 μg/mg (dry weight) $\times$ k at temperature indicated (for both strains).

[c] Expressed as micrograms of tyrosine per milligram of cells per hour. Data from Fig. 4.

These studies indicate that controls for tyrosine synthesis exist which are responsive to various environmental conditions and which are independent of specific tyrosine feedback regulation. The effect of these changed conditions on the tyrosine lost into the growth medium can be correlated with the relative activity and level of prephenate dehydrogenase in the cell. Both the excess tyrosine excreted by NP 164 and the amount present in its pools implicate the major role of tyrosine feedback control on the normal regulation of tyrosine biosynthesis. The operation of other cellular restraints on tyrosine synthesis, functioning in both strains, serves to restrict the loss of this amino acid in mutants such as NP 164 and illustrates the multiple controls which exist to govern amino acid biosynthesis and utilization.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service training grant 1-701-GM-01459-01 from the National Institute of General Medical Sciences and by research grant AM 13105 from the National Institute of Arthritis and Metabolic Diseases.

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