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D-Tyrosine as a Metabolic Inhibitor of *Bacillus subtilis*

W. SCOTT CHAMPNEY[1] AND ROY A. JENSEN

Department of Biology, State University of New York at Buffalo, Buffalo, New York 14214, and Department of Microbiology, Baylor University College of Medicine, Houston, Texas 77025

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The D-isomer of tyrosine is a potent inhibitor of growth in transformable strain 168 of *Bacillus subtilis*. A D-tyrosine-resistant mutant of the inhibited strain was isolated which excreted L-tyrosine, had a diminished growth rate, and required L-phenylalanine to attain the growth rate of the wild-type parent. Mapping by deoxyribonucleate transformation located this resistance in the gene coding for prephenate dehydrogenase. This enzyme in the D-tyrosine-resistant mutant was insensitive to the usual feedback inhibition exerted by L-tyrosine in extracts of strain 168. In contrast, the growth of poorly transformable strain 23 of *B. subtilis*, as well as that of several other *Bacillus* species, was not affected by the analogue. Transformation mapping demonstrated no linkage of this latter "natural resistance" to several different aromatic markers. Prephenate dehydrogenase in extracts from strain 23 was as sensitive as that from strain 168 to feedback inhibition by L-tyrosine in vitro. The relationships of the latter results to the regulation of tyrosine biosynthesis and the possible nature of strain differences in D-tyrosine sensitivity are discussed.

The regulation of aromatic amino acid biosynthesis in different microorganisms has been the subject of extensive investigation (12, 19). In many of these studies, structural analogues of natural amino acids have been used to assess the role and importance of the natural compound in the regulation of cellular activities. In *Bacillus subtilis*, most analogues of L-tyrosine (L-Tyr) inhibit growth by acting as antimetabolites of L-phenylalanine (L-Phe) (R. Guthrie, *personal communication*). In transformable strain 168 of *B. subtilis*, however, the D-isomer of Tyr inhibits growth through its action as a specific antagonist of L-Tyr (W. S. Champney and R. A. Jensen, Bacteriol. Proc., p. 135, 1968).

Previous studies on the regulation of aromatic biosynthesis in this organism have identified L-Tyr as an important regulatory metabolite promoting both feedback inhibition (25) and the repression of enzyme synthesis (26). This investigation describes several characteristics of a mutant of strain 168 which is resistant to D-Tyr and the consequent effects on the regulation of L-Tyr biosynthesis. Striking differences in strain sensitivities to growth inhibition by the analogue are also revealed.

[1] National Institutes of Health Predoctoral Trainee (GM 01459). Present address: Department of Microbiology, Baylor University College of Medicine, Houston, Tex. 77025.

MATERIALS AND METHODS

Bacterial strains. All *B. subtilis* strains and other *Bacillus* species used are described in Table 1. NP 164, a D-tyrosine-resistant (D-Tyr^R) derivative of NP 40 which excretes L-Tyr, was isolated by plating heavy cell suspensions of NP 40 on minimal agar plates containing 50 μg of D-Tyr per ml. L-Tyr excretors were recognized as large colonies surrounded by halos of background feeding on analogue-containing plates. The excreted L-Tyr reversed the growth inhibition of the sensitive background in the vicinity of the resistant colonies and allowed limited growth. Cells from single colony isolates were purified by repeated streakings on plates supplemented with D-Tyr. L-Tyr excretion by D-Tyr^R mutants was assessed in cross-streaking experiments by observing syntrophism with L-Tyr auxotrophs on minimal plates. These mutants also supported the growth of NP 40 on analogue-containing plates, when both were streaked in parallel.

Growth media. Complete solid medium (BAB), liquid minimal medium (Spizizen's salts, 31), and solid minimal medium (modified Davis, 18) were previously described (16).

Growth experiments. Growth experiments were conducted in one of two ways. For most experiments, 10 ml of medium was added to 125-ml flasks with side arms. After inoculation, growth was monitored by the increase in turbidity, measured with a Klett-Summerson colorimeter equipped with a no. 54 (green) filter. Alternatively, 1-ml samples were removed at appropriate intervals from 125-ml flasks containing 25 ml of

TABLE 1. *Bacterial strains used in growth experiments and in transformations*[a]

No.	Parent strain(s)	Description[b]	Origin
	B. subtilis strains		
NP 46		D-Tyr^S	ATCC 6051
NP 93	BG 23 (2, 6)	D-*tyr*^{R}-*23*	Spontaneous revertant
NP 40	168(2, 6)	D-*tyr*^{S}-*168*	Spontaneous revertant
NP 164	NP 40	D-*tyr*^{R}-*168*	Spontaneous mutant
NP 10	SB 70—× SB98[c]	*trp*C *his*-2 *tyr*A; D-*tyr*^{S}-*168*	SB 100; Nester et al. (27)
NP 19	168	*trp*C *his*-2; D-*tyr*^{S}-*168*	SB 25; Nester et al. (27)
NP 26	NP 40	*aro*A; D-*tyr*^{S}-*168*	NMG treatment (15)
NP 35	WB 1016	*tyr*A; D-*tyr*^{S}-*168*	E. W. Nester
NP 61	NP 164—× NP 19	*his*-2; D-*tyr*^{R}-*168*	DNA transformation
NP 62	NP 164—× NP 19	*trp*C; D-*tyr*^{R}-*168*	DNA transformation
NP 67	168	*phe*A; D-*tyr*^{S}-*168*	H12; I. Takahashi (32)
NP 329	NP 40—× NP 10	*his*-2 *tyr*A; D-*tyr*^{S}-*168*	DNA transformation
NP 330	SB 155 (27)	*aro*A; D-*tyr*^{S}-*168*	Spontaneous revertant
	Other bacterial strains		
GP 1	*B. subtilis* var. *niger*	D-Tyr^R	ATCC 6455
GP 2	*B. subtilis* var. *atterimus*	D-Tyr^R	ATCC 6460
GP 3	*B. globigii*	D-Tyr^R	R. Altenbern
GP 4	*B. licheniformis*	D-Tyr^R	C. Thorne
EN 1	*Escherichia coli* B	D-Tyr^R	J. Gallant
EN 13	*Serratia marcescens*	D-Tyr^R	W. Spencer
BUCM 1	*Salmonella typhimurium*	D-Tyr^R	R. Wende
BUCM 2	*Aerobacter aerogenes*	D-Tyr^R	R. Wende

[a] Abbreviations: His, histidine; Tyr, tyrosine; Trp, tryptophan; Phe, phenylalanine; Shk, shikimate; DAHP synthetase, 3 deoxy-D-*arabino*-heptulosonic acid-7-phosphate synthetase; IGP synthetase, indole glycerol-3-phosphate synthetase; ATCC, American Type Culture Collection; NMG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

[b] D-*tyr*^{R}-*23* indicates the "natural resistance" to inhibition by D-Tyr of derivatives of strain 23. D-*tyr*^{S}-*168* indicates the "natural sensitivity" to inhibition by D-Tyr of derivatives of strain 168. D-*tyr*^{R}-*168* indicates the mutation to D-Tyr^R found in strain 168. The symbols *tyr*A$^+$, *phe*A$^+$, *aro*A$^+$, and *trp*C$^+$ specify loci for the enzymes prephenate dehydrogenase, prephenate dehydratase, DAHP synthetase, and IGP synthetase, respectively (1, 23).

[c] Notation for genetic crosses from Nester, Schafer, and Lederberg (27).

medium, and the increase in turbidity was followed by measuring the optical density (OD) at 600 nm in a Gilford 240 Spectrophotometer. Incubations were conducted in a New Brunswick gyratory water bath, maintained at 37 C. The duration of most experiments was 8 to 12 hr. All experiments were begun by a dilution of an inoculum of cells which had been grown overnight in minimal medium.

Growth curves are defined in terms of the specific growth rate k (in dimensions of hr^{-1}), the slope of a semilogarithmic plot of culture turbidity against time, during the exponential phase of growth (14). The specific growth rate is useful in comparing cells grown under various experimental conditions.

Genetic analysis. The protocol for deoxyribonucleate transformation was described previously (15). Deoxyribonucleic acid (DNA) was isolated from cells by the method of Marmur (21). Linkage of markers in transformations was analyzed by picking transformants for the selected marker to BAB plates followed by replica plating to appropriately supplemented minimal plates to determine the frequency of joint transfer of the unselected marker(s). Linkage is expressed as the cotransfer index, r (27).

Genetic analysis of D-tyr^R-168. Cross-streaking tests showed that NP 164 excreted less Tyr on tryptophan (Trp) agar than on minimal agar. Hence, particular care was needed to ensure that recombinants were properly scored for D-*tyr*^{R}-*168* whenever *trp*C was an unselected marker in the cross. In the latter case, the presence of Trp tended to inhibit the full expression of the D-*tyr*^{R}-*168* phenotype. In these circumstances (e.g., NP 164 — × NP 62), accurate mapping of the D-*tyr*^{R}-*168* locus required at least two purifications of transformants by transfer onto the selective medium. D-*tyr*^{R}-*168* transformant clones that contained an admixture of recipient D-*tyr*^{S}-*168* cells were especially likely to be scored as D-*tyr*^{S}-*168* in the absence of the purification regimen. The latter results were best scored by reading the replica plates after two days incubation at 37 C, rather than the usual one day. In like manner, when experiments involved the analysis of the linkage of D-*tyr*^{R}-*168* to *aro*A, the *aro*A D-*tyr*^{R}-*168* class of transformants required purification of the transformant clones and an extra day of incubation of replica plates due to the fact that shikimic acid is not sufficiently permeable to the cell (17) to permit the full expression of the cell's ability to overproduce Tyr in the presence of the D-*tyr*^{R}-*168* marker.

In some crosses, one class of single transformant could not be scored easily, e.g., *tyr*A D-*tyr*^{R}-*168*. The value of *r* was approximated by assuming that the frequency of the unscored class of single transformants was equal to the frequency of the other class of singles scored. In practice, the actual frequencies of the two classes of single transformants may vary as much as twofold. For example, in crosses testing cotransfer of *his*-2 and D-*tyr*^{R}-*168*, the frequency of *his*-2 single recombinants exceeded the frequency of D-*tyr*^{R}-*168* single recombinants by a factor of 1.65. Possible explanations for this phenomenon have been elaborated elsewhere (4).

The three point test cross, NP 164 — × NP 329, was done with DNA concentrations which were 100-fold under saturating levels, as determined by control experiments using samples of competent cells from the same competence regimen preserved by freezing in liquid nitrogen. *Tyr*A recombinants were tested for D-Tyr^R by plating heavy lawns of each recombinant clone on minimal agar. The 10 to 50 spontaneous revertants which arose after incubation for 3 days at 37 C were replica plated to D-Tyr plates, and growth in the presence of the analogue was confirmed.

Extract preparation. Extracts for enzyme analysis were made from 200 ml of minimal-grown cells shaken at 37 C. The cells were pelleted at 3,000 × *g* at 4 C, resuspended in 5.0 ml of potassium phosphate buffer (0.10 M, *p*H 7.8) containing 0.1 M KCl, and broken by 15 min of treatment with lysozyme (100 μg/ml) at 37 C. Deoxyribonuclease and ribonuclease at a final concentration of 10 μg/ml were added to digest nuclei acids. Cell debris was removed by centrifugation at 12,000 × *g* for 15 min at 4 C, and 4 ml of the clear supernatant fluid was passed through a Sephadex G-25 (coarse) column (1.2 × 15 cm). The leading 3 ml of eluate was collected.

Substrate preparation and enzyme assays. Barium prephenate was isolated by the method of Gibson (13). This substrate was 75% pure, based on the acid conversion of prephenate to phenylpyruvic acid by the use of an extinction coefficient of 17,500 L/mole·cm at 320 nm in 1 N NaOH (10). Prephenate concentrations have been corrected for purity. *p*-Hydroxyphenylpyruvic acid, formed from prephenate in the presence of nicotinic adenine dinucleotide (NAD) and a suitable cell extract, was assayed by the Millon test as modified by Schwinck and Adams (30). The reaction mixture contained: NAD, 1.0 μmole; potassium phosphate (*p*H 7.8), 40 μmoles; tris(hydroxymethyl)aminomethane hydrochloride buffer (*p*H 8.1), 4 μmoles; potassium chloride, 40 μmoles; and variable amounts of barium prephenate and Tyr, in a final volume of 0.5 ml. Enzyme incubations were carried out in 13 by 100-mm test tubes for 20 min in a reciprocal shaker at 37 C. Substrate blanks and Tyr blanks were included as controls, and the resulting absorbance was subtracted from the optical density measured at 490 nm in a DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Prephenate dehydrogenase activity was also measured by monitoring the increase in fluorescence of reduced nicotinamide adenine dinucleotide formed in the reaction by use of an Aminco-Bowman Spectrophotofluorometer (American Instrument Company, Silver Spring, Md.). The excitation wavelength was 340 nm; the emission wavelength was 460 nm. The reaction mixture (1.0 ml) was maintained at 37 C in a temperature-controlled cuvette holder, and the reaction was observed by means of a linear recorder. Comparable results were obtained with either assay procedure.

Protein concentrations were determined by the method of Lowry et al. (20), with bovine serum albumin as a standard.

Chemicals. D-Tyr was purchased from Calbiochem, Los Angeles, Calif., and was >99% free from L-Tyr contamination (manufacturer's analysis based on optical rotation). Nucleases were also purchased from Calbiochem. *p*-Hydroxyphenylpyruvic acid (used as a standard), NAD, 3X crystalline lysozyme, and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. Other chemicals purchased commercially were of the highest quality available.

RESULTS

The growth of *B. subtilis* strain 168 was strongly inhibited by the stereoisomeric analogue, D-Tyr. Supplementation of minimal agar plates with D-Tyr at 50 μg/ml totally prevented colony formation in NP 40. Prolonged incubation at 37 C of a lawn of about 10^7 cells on the same medium gave rise ultimately only to analogue-resistant mutants. In a liquid minimal-glucose medium, wild-type NP 40 had a specific growth rate, *k*, of 0.53 hr^{-1}, doubling every 78 min (Fig. 1). D-Tyr at 100 μg/ml reduced the rate 80%, to 0.095 hr^{-1}. The D-isomer acted as a specific antimetabolite of L-Tyr in *B. subtilis*. L-Tyr at 10 μg/ml partially reversed the D-Tyr inhibition, giving a *k* value of 0.24 hr^{-1}, a rate about half of that observed in the absence of the analogue. Higher concentrations of L-Tyr completely reversed the inhibition of growth in liquid medium. In spite of the structural similarity of L-Phe to L-Tyr, the former compound reversed the inhibition poorly even at concentrations of 25 μg/ml or more. Neither L-Trp nor the nonaromatic amino acids reversed the inhibition by D-Tyr. Figure 2 indicates the inhibition of the growth rate of NP 40 in minimal medium as a function of the concentration of D-Tyr. Three μg of the analogue per ml were sufficient to inhibit the growth rate by 50%. The inset shows that 500 μg of D-Tyr per ml allowed no growth for at least 12 hr.

As detailed in Table 2, only *B. subtilis* NP 40, and the American Type Culture Collection strain NP 46, were sensitive to growth inhibition by D-Tyr. Inhibition of growth was analyzed both on analogue-containing agar plates and in liquid minimal medium. The growth of *B. subtilis* NP 93, a derivative of the poorly transformable strain 23, was unaffected by the analogue. The latter

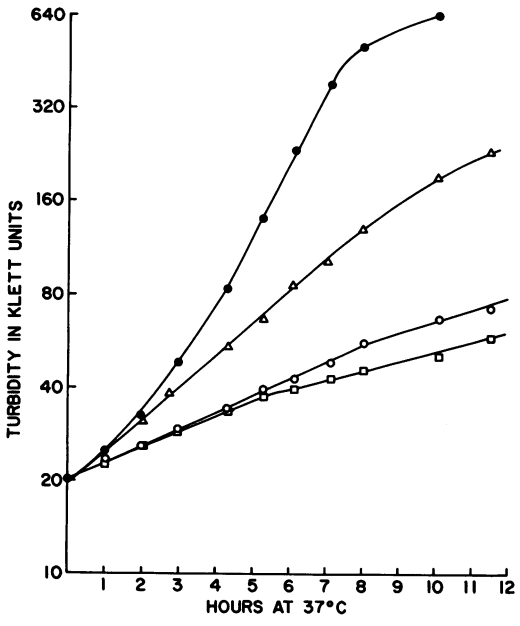

FIG. 1. *Effect of* L-*Phe and* L-*Tyr upon inhibition of B. subtilis NP 40 by 100 μg of* D-*Tyr per ml. Symbols:* ●, *uninhibited growth of control in minimal-glucose medium* (k = 0.53 hr^{-1}); □, *inhibited growth in minimal-glucose medium with 100 μg of* D-*Tyr per ml* (k = 0.095 hr^{-1}); △, *growth in minimal-glucose medium with 100 μg of* D-*Tyr per ml and 10 μg of* L-*Tyr per ml* (k = 0.24 hr^{-1}); ○, *growth in minimal-glucose medium with 100 μg of* D-*Tyr per ml and 25 μg of* L-*Phe per ml* (k = 0.13 hr^{-1}). *Ordinate: culture turbidity expressed as Klett units. Abscissa: growth time in hours at 37 C.*

resistance to growth inhibition by D-Tyr was also characteristic of the *B. subtilis* varieties *niger* and *atterimus*, and the other *Bacillus* species, as well as the enteric genera examined. In Fig. 3, the growth rates of NP 40 and NP 93 in the presence of 100 μg of D-Tyr per ml are compared. In marked contrast to NP 40, strain NP 93 grew equally well in the presence or absence of the analogue. Growth of NP 93 in the presence of the analogue was unaffected by the addition of L-Tyr or L-Phe. Cross-feeding tests and enzymological studies (described below) indicated that the D-Tyr^R phenotype of NP 93 was not due to a lack of regulation of L-Tyr synthesis and the attendant excretion of this amino acid.

Mutants of NP 40, resistant to the analogue, were isolated as described in Materials and Methods. These mutants overproduced L-Tyr and excreted it into the medium, presumably because they were no longer responsive to one or more of the regulatory effects exerted by L-Tyr. Of several such mutants, NP 164 was selected for further study. When grown in minimal medium with or without D-Tyr, this mutant had a k-value of 0.34 hr^{-1} (120-min doubling time), a rate which was 65% of that characteristic of the parental NP 40 (Fig. 4). The addition of L-Tyr did not influence the growth rate of NP 164, but L-Phe supplementation stimulated the value of k about 25% to 0.41 hr^{-1}. A comparable stimulation of the growth rate in minimal medium also occurred in the presence of 100 μg of shikimic acid per ml, a common precursor in the biosynthesis of Phe and Tyr.

Mapping of the loci coding for D-Tyr^R in NP 93 and NP 164 was carried out by DNA transformation. Transforming DNA was isolated from NP 40, NP 93, and NP 164. Figure 5 shows the map location of the D-Tyr^R locus of NP 164 (D-*tyr*^{R}-*168*). The genetic crosses used in the transformation protocols are detailed in Table 3. D-*tyr*^{R}-*168* mapped at *tyr*A (r = 0.92), the gene specifying the enzyme prephenate dehydrogenase (23). The three-point test cross shown at the bottom of Table 3 placed the D-*tyr*^{R}-*168* locus immediately to the left of *tyr*A. A second three-point test cross (R. A. Jensen, *unpublished data*) with a *trp*C *tyr*A recipient also placed D-*tyr*^{R}-*168* to the left of *tyr*A.

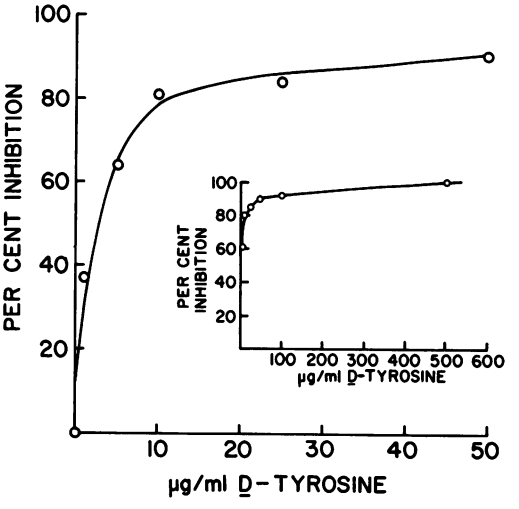

FIG. 2. *Inhibition of growth rate by* D-*Tyr in B. subtilis NP 40. Ordinate: per cent inhibition of specific growth rate, k, of wild type. Abscissa: final* D-*Tyr concentration in growth vessel, in μg/ml. The inset presents the inhibition results with a highly compressed abscissa to show that inhibition approaches 100% at high concentrations of* D-*Tyr. Cells were grown at 37 C for 12 hr in minimal-glucose medium containing* D-*Tyr at the final concentrations indicated.*

TABLE 2. *D-Tyr sensitivities of various Bacillus species and enteric bacteria*[a]

Organism	No.	Colony formation	Specific growth rate (hr^{-1})	
			Without D-Tyr	With D-Tyr
B. subtilis	NP 40	—	0.530	0.095
B. subtilis	NP 164	+++	0.340	0.340
B. subtilis	NP 93	+++	0.530	0.520
B. subtilis (ATCC 6051)	NP 46	+	0.815	0.264
B. subtilis var. *niger*	GP 1	+++	0.570	0.520
B. subtilis var. *atterimus*	GP 2	+++	0.625	0.625
B. globigii	GP 3	+++	0.525	0.515
B. licheniformis	GP 4	+++	0.615	0.620
E. coli B	EN 1	+++	0.960	0.925
S. marcescens	EN 13	+++	0.620	0.615
S. typhimurium	BUCM 1	+++	0.850	0.845
A. aerogenes	BUCM 2	+++	1.140	1.090

[a] Colony formation was examined on minimal agar plates supplemented with 50 μg of D-Tyr per ml. Specific growth rates were calculated after growth in liquid minimal-glucose medium with or without 100 μg of D-Tyr per ml. Both vessels received an identical inoculum of minimal grown cells. Symbols: —, complete inhibition of colony formation after incubation at 37 C for 2 days; +, only small colonies developed after incubation; +++, no inhibition of colony formation after incubation.

[b] See Table 1 for a complete description of strains.

When DNA isolated from NP 93 was used with the preceding genetic recipient strains, however, no linkage of D-*tyr*^{R}-*23* could be demonstrated with *tyr*A. Since *tyr*A is the general aromatic linkage group of *B. subtilis* (27), the D-Tyr^R of NP 93 is unlinked to this gene cluster. Table 4 also indicates no linkage of D-*tyr*^{R}-*23* to *phe*A or to *aro*A, markers which are linked neither to the major aromatic linkage group nor to one another. The genetic location of the locus specifying the "natural D-Tyr^R" of NP 93 has not yet been established.

Genetically different bases for D-*tyr*^{R}-*23* of NP 93 and D-*tyr*^{R}-*168* of NP 164 were confirmed by enzyme analyses of prephenate dehydrogenase isolated from these two strains and from NP 40. Figure 6 shows a substrate saturation curve of prephenate dehydrogenase obtained with an extract from NP 40. The enzyme exhibited a typical hyperbolic saturation curve with a K_m value of about 0.25 mM prephenate. L-Tyr, at a fixed concentration of 0.008 mM, competitively inhibited activity, producing a sigmoidal saturation curve. D-Tyr concentrations greater than 0.10 mM were required for significant inhibition. In contrast to the inhibition exerted by L-Tyr, D-Tyr appeared to be a noncompetitive inhibitor. The substrate inhibition observed in the control reaction mixture was potentiated in the presence of either Tyr isomer. Comparable substrate saturation curves were obtained in extracts prepared from NP 93. The enzyme from this strain was similarly susceptible to inhibition by either D- or L-Tyr, and its specific activity was approximately that observed for the enzyme from NP 40. In contrast to its effect in *B. subtilis*, D-Tyr is a poor inhibitor of prephenate dehydrogenase activity in *Aerobacter aerogenes* (11); only L-Tyr is an effective inhibitor of the enzyme in this species.

As expected by its excretion of L-Tyr and the genetic location of D-*tyr*^{R}-*168*, the mutant NP 164 proved to be altered in its sensitivity to feedback inhibition of prephenate dehydrogenase by L-Tyr. As Fig. 7 shows, the sigmoidal substrate saturation curve characteristic of NP 40 in the presence of L-Tyr (Fig. 6) was abolished in NP 164. Inhibition of enzyme activity by either compound occurred only at high substrate concentrations. Apparently, L-Tyr potentiates a substrate inhibition in NP 164 similar to the effect observed in NP 40. A possible physiological significance of this observation is discussed below. The desensitization of the enzyme in NP 164 is perhaps most obviously displayed by a comparison of the inhibition curves of Fig. 8. L-Tyr at 0.05 mM was sufficient to inhibit enzyme activity almost 100% in NP 40, whereas the same concentration of L-Tyr in the D-Tyr^R mutant NP 164 had no inhibitory effect. A comparison of Fig. 6 and 7 reveals a fourfold decrement in the specific activity of prephenate dehydrogenase from NP 164 relative to NP 40, indicating the repressive effects of the L-Tyr overproduced by the mutant.

DISCUSSION

The importance of L-Tyr as a key regulatory compound in *B. subtilis* metabolism has been documented by several studies. Nester and Jensen

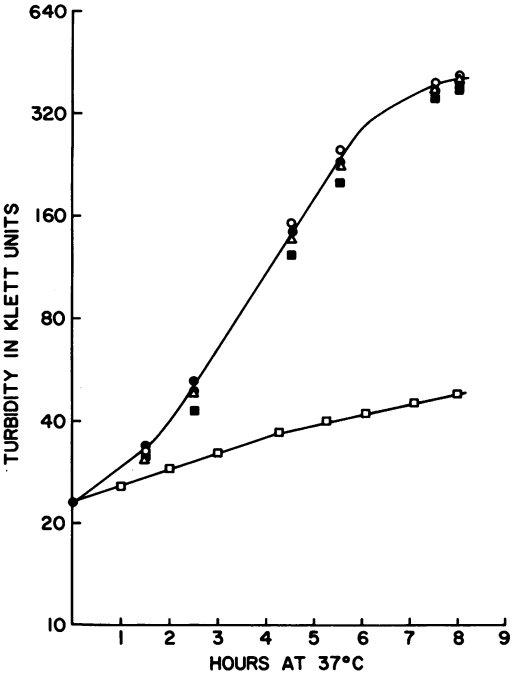

FIG. 3. *Insensitivity of NP 93 to growth inhibition by* D-*Tyr. Ordinate and abscissa are as given in Fig. 1. Symbols:* ■, *NP 93 in minimal-glucose with* D-*Tyr at 100 μg/ml (k = 0.52 hr⁻¹);* △, *NP 93 in minimal-glucose with* D-*Tyr at 100 μg/ml and* L-*Tyr at 10 μg/m (k = 0.53 hr⁻¹);* ○, *NP 93 in minimal-glucose with* D-*Tyr at 100 μg/ml and* L-*Phe at 25 μg/ml (k = 0.53 hr⁻¹);* ●, *NP 40 in minimal-glucose (k = 0.51 hr⁻¹);* □, *NP 40 in minimal-glucose with* D-*Tyr at 100 μg/ml (k = 0.095 hr⁻¹, from Fig. 1). The growth rate of NP 93 in minimal-glucose was identical with that in minimal-glucose with 100 μg of* D-*Tyr per ml (Table 2). For clarity, a single curve is used to represent the similar growth rates observed under the first four conditions.*

(25) demonstrated that L-Tyr was a sensitive inhibitor of prephenate dehydrogenase activity, 0.023 mM L-Tyr inhibiting 50% at a prephenate concentration of 2.5 mM. L-Tyr also exerts repressive control over the biosynthesis of prephenate dehydrogenase and other enzymes of the aromatic pathway (26). The inhibition of the growth of NP 40 by D-Tyr and its specific reversal by L-Tyr indicates that the analogue mimics some function of the natural amino acid. The isolation of mutants resistant to the analogue (W. S. Champney and R. A. Jensen, Bacteriol. Proc., p. 135, 1968) permitted a more extended examination of the importance of L-Tyr in the regulation of its own biosynthesis. Since certain D-*tyr*^{R}-*168* mutants were found to overproduce L-Tyr, the mutation involved a relaxation in the control of L-Tyr synthesis. This conclusion was also reached by Polsinelli (28), who isolated Tyr-excreting mutants of *B. subtilis* which were resistant to 700 μg of 3-aminotyrosine per ml. These analogue-resistant mutants also mapped at the *tyr*A locus.

The observation that L-Phe, but not L-Tyr, stimulated the basal growth rate of NP 164 suggested that a perturbation in the regulation of Tyr biosynthesis had also disturbed the normal synthesis of Phe, resulting in a level of this amino

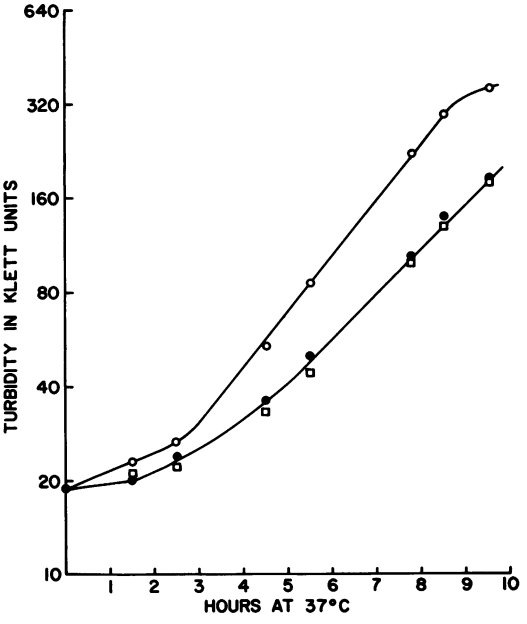

FIG. 4. *Growth characteristics of NP 164. Ordinate and abscissa are as given in Fig. 1. Symbols:* ●, *NP 164 in minimal-glucose (k = 0.34 hr⁻¹);* □, *NP 164 in minimal-glucose with 100 μg of* D-*Tyr per ml (k = 0.34 hr⁻¹);* ○, *NP 164 in minimal-glucose with* L-*Phe at 100 μg/ml (k = 0.41 hr⁻¹). For clarity, a single curve is used to represent the similar growth rates observed under the first two conditions.*

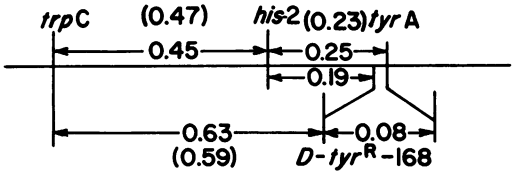

FIG. 5. *Map location of the* D-*tyr*^{R}-*168 locus within the B. subtilis linkage group of aromatic loci. Genetic crosses were conducted as previously described (15). Numbers between the arrows indicate the map distance, q, separating genetic markers. Values given in parentheses denote the q values obtained previously (27). Map distance, q, and cotransfer index, r, were previously defined (27).*

TABLE 3. *Genetic mapping of* D-*tyr*^{R}*-168 in NP 164*[a]

DNA donor	Recipient	Cotransfer frequencies	*r*
NP 40	NP 61	194/218 His^+ were D-Tyr^S	0.81[b]
NP 40	NP 62	162/300 Trp^+ were D-Tyr^S	0.37[c]
NP 164	NP 35	231/241 Tyr^+ were D-Tyr^R	0.92[d]
NP 164	NP 19	177/250 Trp^+ were His^+	0.55[e]
NP 164	NP 329	787/787 Tyr^+ His^+ were D-Tyr^R 0/502 Tyr^- His^+ were D-Tyr^R[f]	

[a] Genetic crosses were made as described in Materials and Methods. The D-*tyr*^{R}*-168* locus mapped at *tyr*A; *r* = 0.92. See Table 1 for further information about mutant characteristics.
[b] Frequency of *his*-2 D-*tyr*^{S}*-168* transformant class assumed equal to *his*-2^+ D-*tyr*^{R}*-168*.
[c] Frequency of *trp*C D-*tyr*^{S}*-168* transformant class assumed equal to *trp*C^+ D-*tyr*^{R}*-168*.
[d] Frequency of *tyr*A D-*tyr*^{R}*-168* transformant class assumed equal to *tyr*A^+ D-*tyr*^{S}*-168*.
[e] A total of 250 transformants of all recombinant classes (*his*-2^+ *trp*C^+, *his*-2 *trp*C^+, *his*-2^+ *trp*C) were tested.
[f] After reversion of *tyr*A, since the presence of L-Tyr masks the D-Tyr^S phenotype.

TABLE 4. *Genetic mapping of* D-*tyr*^{R}*-23 in NP 93*[a]

DNA donor	Recipient	Cotransfer frequencies	*r*
NP 93	NP 35	10/555 Tyr^+ were D-Tyr^R	0.009[b]
NP 93	NP 67	8/210 Phe^+ were D-Tyr^R	0.020[c]
NP 93	NP 26	4/341 Shk^+ were D-Tyr^R	0.006[d]
NP 93	NP 330	3/259 Shk^+ were D-Tyr^R	0.006[d]

[a] Genetic crosses were made as described in Materials and Methods. No linkage was found between D-*tyr*^{R}*-23* and three different genetic regions for aromatic loci on the *B. subtilis* chromosome.
[b] Frequency of *tyr*A D-*tyr*^{R}*-23* transformant class assumed equal to frequency of *tyr*A^+ D-*tyr*^{S}*-168*.
[c] Frequency of *phe*A D-*tyr*^{R}*-23* transformant class assumed equal to frequency of *phe*A^+ D-*tyr*^{S}*-168*.
[d] Frequency of *aro*A D-*tyr*^{R}*-23* transformant class assumed equal to frequency of *aro*A^+ D-*tyr*^{S}*-168*.

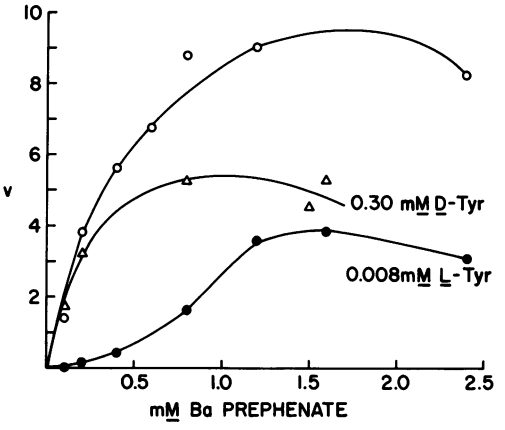

FIG. 6. *Substrate saturation curve of prephenate dehydrogenase isolated from NP 40. Ordinate: nanomoles of p-hydroxyphenylpyruvic acid per minute per milligram of protein. Abscissa: prephenate concentration,* mM. *Symbols:* ○, *(control) enzyme activity;* ●, *enzyme activity in presence of 0.008* mM L-*Tyr;* △, *enzyme activity in presence of 0.30* mM D-*Tyr. Assays were performed as described in Materials and Methods. The reaction mixture contained 0.08 mg of protein.*

acid insufficient to support the normal growth rate. This interpretation is consistent with the fact that shikimic acid, an intermediate in the synthesis of both Tyr and Phe, will substitute for Phe as a growth stimulant. A partial Phe requirement is also reflected by the increased sensitivity of NP 164 to growth inhibition by the Phe analogue, β-2-thienylalanine, compared to its parent NP 40 (Champney and Jensen, *unpublished data*). L-Tyr overproduction in NP 164 could lead to the observed Phe requirement by depleting prephenic acid, the common substrate, or by "cross-inhibiting" prephenate dehydratase, the first enzyme specific to the synthesis of L-Phe (25).

Genetic mapping of the D-*tyr*^{R}*-168* locus of NP 164 by DNA transformation revealed a second fact relating to L-Tyr regulation. The map location of the resistance locus at *tyr*A, the gene for prephenate dehydrogenase, strongly implicated this enzyme as a key regulatory protein. Since the mutation to D-Tyr^R led concomitantly to L-Tyr overproduction, the genetic data suggested either an alteration in the allosteric recognition site of the enzyme or its constitutive biosynthesis. This latter possibility was dismissed since the specific activity of prephenate dehydrogenase from NP 164 was, in fact, fourfold lower than that of wild-

type NP 40 (*compare* Fig. 6 and 7). Similarly, a threefold repression by L-Tyr was observed by Nester et al. (26) for prephenate dehydrogenase isolated from *B. subtilis* 168 grown on L-Tyr and in a mutant resistant to 3-fluorotyrosine. The repressive control of prephenate dehydrogenase

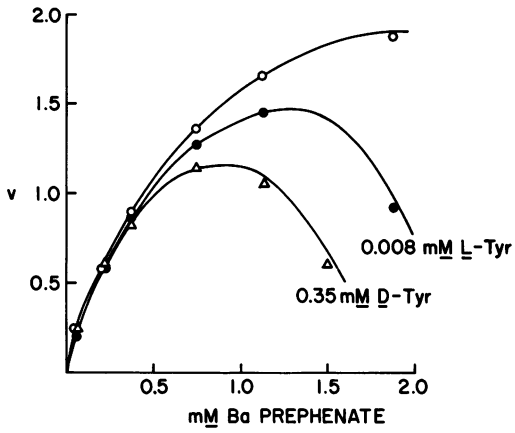

FIG. 7. *Substrate saturation curve of prephenate dehydrogenase isolated from NP 164. Ordinate and abscissa as given in Fig. 6. Symbols: ○, control enzyme activity; ●, enzyme activity in presence of 0.008 mM L-Tyr; △, enzyme activity in presence of 0.35 mM D-Tyr. The reaction mixture contained 0.5 mg of protein.*

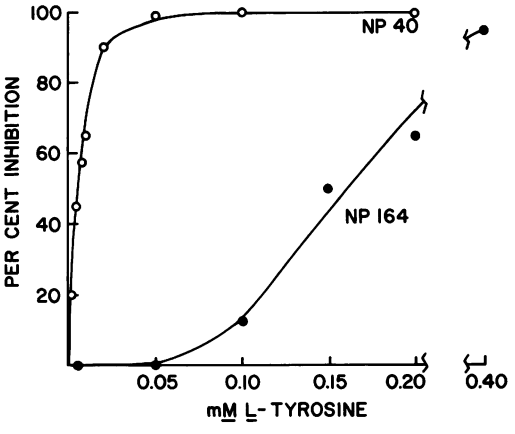

FIG. 8. *Inhibition curves of prephenate dehydrogenase activities isolated from NP 40 and NP 164. Ordinate: per cent inhibition of control enzyme activity* $= (v_0 - v_i)/v_0$. *Abscissa: L-Tyr concentration, mM. Symbols: ○, L-Tyr inhibition of activity of enzyme isolated from NP 40; ●, L-Tyr inhibition of activity of enzyme isolated from NP 164. The reaction vessels contained 1.6 mM barium prephenate and 0.08 mg of protein from an extract of NP 40 or 1.2 mM barium prephenate and 0.5 mg of protein from an extract of NP 164.*

synthesis is not a sufficient regulatory device in *B. subtilis*, however, since L-Tyr overproduction can occur under conditions of repressed enzyme formation, in the absence of control by feedback inhibition. This emphasizes the importance of L-Tyr-mediated feedback inhibition in the regulation of Tyr biosynthesis in *B. subtilis* 168.

The distinct Tyr sensitivity of prephenate dehydrogenase isolated from NP 40 substantiated the preceding observations concerning the role of L-Tyr as a feedback modulator of this enzyme. The competitive inhibition of activity by L-Tyr in NP 40 indicates an altered substrate affinity in the presence of this inhibitor and is in agreement with the kinetic results obtained by Nester (24). The sigmoidal substrate saturation curve observed for the enzyme of NP 40 in the presence of L-Tyr is characteristic of regulatory enzymes of the K system type and implies cooperativity of substrate binding in the presence of the modulator (22). However, we find that both the apparent K_m for prephenate and the apparent V_{max} attained are influenced by L-Tyr, suggesting a dual effect of this compound. Thus L-Tyr is a competitive inhibitor of activity at low substrate levels, whereas it potentiates a substrate inhibition at high prephenate concentrations. Inhibition of activity at high prephenate concentrations has also been demonstrated for the enzyme in extracts from *Escherichia coli* (30). The particular sensitivity of L-Tyr as a metabolic signal for this enzyme implicates a substantial potential for delicate regulation in vivo. The distinctly different kinetics of inhibition of the enzyme by D-Tyr may reflect (i) its poor ability to mimic L-Tyr as a specific inhibitor (since only the V_{max} is altered in the presence of the analogue) and (ii) its inhibitory action exerted at a different allosteric site, such as that promoting inhibition by L-Phe (24, 25), or both.

The mutation to analogue resistance in NP 164 resulted in a loss of feedback sensitivity of prephenate dehydrogenase to L-Tyr, a conclusion apparent from an inspection of the altered substrate saturation and inhibition curves of the enzyme from this mutant (Fig. 7 and 8). These figures also illustrate the dual effect of L-Tyr on the activity of the enzyme. Thus, the mutation to analogue resistance resulting in an insensitivity to L-Tyr inhibition at low substrate concentrations has *not* simultaneously affected the ability of L-Tyr to potentiate a substrate inhibition at high prephenate levels. The site for the L-Tyr-mediated substrate inhibition was not altered by the mutation NP 164, and presumably accounts for the inhibition of the activity of prephenate dehydrogenase at high Tyr concentrations (Fig. 8). The altered susceptibility to substrate inhibition in the

presence of L-Tyr may be of physiological significance. The inhibition of enzyme activity by high concentrations of prephenate in the presence of L-Tyr may reflect a mechanism for diverting excess prephenate into Phe synthesis. D-Tyr apparently mimics L-Tyr in this respect, as Fig. 6 and 7 indicate. Supporting this suggestion is the observation that NP 164 will grow at 65% of the wild-type rate in the absence of exogenous Phe, indicating some synthesis of this compound. However, this shunt mechanism is not sufficient in NP 164, since its growth is limited by the endogenous rate of Phe synthesis. It is also known (9) that prephenate dehydratase, the first Phe-specific enzyme, is not subject to substrate inhibition by prephenate in *B. subtilis* 168.

The simplest explanation consistent with the present results is that, in NP 40, D-Tyr competes with L-Tyr either for activation by the tyrosyl-transfer ribonucleic acid (tRNA) synthetase, entry into cellular proteins, or both. Presumably, D-Tyr inhibits the activity of prephenate dehydrogenase in vivo, thus reducing the amount of L-Tyr made and available for protein synthesis. The increased ratio of D- to L-Tyr would allow the analogue to be activated or to enter proteins in excess of the natural isomer (29). The studies of Calendar and Berg (7, 8) showed that in *B. subtilis* 168, D-Tyr can be activated by the tyrosyl-RNA activating enzyme in vitro. The V_{max} of synthetase enzyme in the presence of D-Tyr was 10% of that found with L-Tyr as a substrate, whereas the K_m values differed only by a factor of three. Competition between the Tyr isomers for this enzyme might be expected since the affinity constants are similar. That Tyr analogues can be incorporated into proteins in *B. subtilis* has been shown by Aronson and Wermus (3) by the use of *m*-tyrosine [DL-β(3-hydroxyphenyl)alanine], a Phe antagonist. The analogue resistance of NP 164 would, therefore, result from a more favorable ratio of L- to D-Tyr owing to the overproduction of the former compound in the mutant. The loss of L-Tyr feedback sensitivity gives the excess L-Tyr formed a competitive advantage over D-Tyr, and would thus allow for growth in the presence of the analogue.

The varied effect of D-Tyr upon the growth of distinctive strains of *B. subtilis* reveals obvious differences in their L-Tyr metabolism or regulation. The separate chromosomal locations of D-*tyr*^{R}-*168* and D-*tyr*^{R}-*23* indicate a fundamental difference in the genetic basis for resistance in these two strains. This variation in D-Tyr sensitivity is but one of several characteristics known to distinguish strains 168 and 23 of *B. subtilis* (2, 25). The D-Tyr^R of NP 93 may result from (i) an impermeability to D-Tyr; (ii) a D-Tyr racemase activity, similar to the L-alanine racemase in *B. subtilis* described by Berberich et al. (5); (iii) an altered tyrosyl-RNA activating enzyme which may not recognize the analogue as it apparently does in strain 168 (8); or (iv) possible variations in the levels (specific activities) of certain key enzymes in the aromatic pathway, resulting in altered enzyme amounts or substrate concentrations. Whatever the exact explanation for the strain differences, the observation that differences in sensitivity to the analogue exist between distinct strains of the same bacterial species, together with the ability to produce interstrain exchanges of the genes responsible for the differences by DNA transformation, offers attractive experimental material for the study of regulatory interactions in related strains bearing subtle differences in their evolutionary histories.

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ADDENDUM IN PROOF

Recently, Calendar and Berg (J. Mol. Biol., **26**: 39–54, 1967) have shown that D–Tyr is incorporated into proteins by the use of an in vitro protein–synthesizing system, with *E. coli* ribosomes and supernatant enzymes from *E. coli* or *B. subtilis*. *E. coli*, but not *B. subtilis* 168, had a D–tyrosyl–tRNA deacylase activity. This activity, if present in *B. subtilis* strain 23, could explain its natural resistance to D–Tyr.

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