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Molecular Basis for the Differential Anti-Metabolite Action of D-Tyrosine in Strains 23 and 168 of *Bacillus subtilis*

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Summary. The basis for the difference between strains 168 (D-tyrosine-sensitive) and 23 (D-tyrosine-resistant) of *Bacillus subtilis* at the molecular level is that of transport of D-tyrosine into the cell. Strain 23 does not incorporate significant amounts of D-tyrosine into whole cells. A mutant derivative was isolated from strain 23 which had an altered transport system permitting D-tyrosine uptake, a change which also led to inhibition of growth by D-tyrosine. Strain 168 is extremely sensitive to growth inhibition caused by low concentrations of the D-isomer of tyrosine. A mutant derivative of strain 168 selected for its D-tyrosine resistant phenotype had an altered transport system which no longer recognized the D-isomer of tyrosine. These mutants define at least one element of the tyrosine transport system in *B. subtilis* and provide a convenient phenotype for the eventual location of the chromosal map position.

The broad distribution and possible metabolic roles of D-amino acids in nature have been reviewed recently (Corrigan, 1969). D-amino acids are normal constituents of certain bacterial structures such as cell wall and capsular material (Bovarnick, 1942; Salton and Williams, 1954). The erroneous replacement by the D-isomer of a given amino acid for the L-isomer in most cellular proteins would provoke drastic physiochemical effects owing to stereochemical perturbations. Nevertheless, D-isomers are not generally inhibitory to the growth of microbial cells in contrast to the common inhibitory effects of many synthetic analogues of L-amino acids. Accordingly, stereo-selective mechanisms commonly exist in cells which discriminate against D-amino acids. Still other mechanisms of resistance include the activities of amino acid racemases, the specific degradation or utilization (e. g., for cell wall synthesis) of exogenous D-amino acids, or the degradation of a D-amino acid-tRNA complex by deacylase activity (Calendar and Berg, 1967).

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In exceptional cases, exogenous D-amino acids may inhibit the growth of certain sensitive bacteria, and this is usually attended by obvious morphological alterations. These effects include noticeable cell enlargement and inhibition of cell division as observed in *Erwinia* species in the presence of either D- or DL-amino acids (Grula, 1960). The general inhibitory effect of many D-amino acids noted in species of *Erwinia* is not observed in *Bacillus subtilis*, but the D-isomer of tyrosine does lead to the failure of cells to separate, exaggerated chain formation, and growth inhibition in *B. subtilis* (Champney and Jensen, 1969, 1970a). D-tyrosine has also been reported to inhibit the growth of *Neurospora crassa*, an eucaryote (Horowitz *et al.*, 1970).

The strain of *B. subtilis*, widely used for genetic studies (strain 168), is extremely susceptible to growth inhibition by D-tyrosine. The molecular events associated with the inhibition of growth by D-tyrosine in this strain include the false feedback inhibition (and probably false repression) of prephenate dehydrogenase, a key regulatory enzyme of L-tyrosine biosynthesis (Champney and Jensen, 1970b). These molecular events decrease the intracellular concentration of L-tyrosine, thereby allowing the increased formation of D-tyrosine-containing proteins. Accordingly, the growth inhibition by D-tyrosine is progressively more severe as a function of time, as is the inhibition of the synthesis of cellular macromolecules and the increase in viable count. Isotopic D-tyrosine was shown to be incorporated into cellular proteins without a change of optical configuration (Champney and Jensen, 1970a). The incorporation of D-tyrosine into protein during growth was consistent with the *in vitro* findings of Calendar and Berg (1967).

In contrast, a closely related strain of *B. subtilis* (strain 23) is totally resistant to the presence of D-tyrosine in the growth environment. We know of no species of bacteria other than strain 168 of *B. subtilis* and *B. subtilis* ATCC 6051 (Champney and Jensen, 1969) which are growth inhibited by D-tyrosine. Likewise we know of no other D-amino acid which exerts as potent an effect upon growth (D-tyrosine inhibits colony formation in strain 168 at concentrations as low as 0.1 μg per ml). Since strains 168 and 23 are so widely used and because many laboratory strains are genetic hybrids of these two strains (Kane *et al.*, 1971), it was of interest to determine the molecular basis for the strain difference with respect to D-tyrosine inhibition of growth.

Materials and Methods

Bacterial Strains. Information about the *B. subtilis* strains used is given in Table 1.

Culture Media. Nitrogen deficient minimal medium refers to minimal salts-glucose medium lacking $(NH_4)_2SO_4$, the only nutritional source of nitrogen. The

Table 1. Strains of *Bacillus subtilis*

Collection No.	Description[a]	Parent strain	Mutational origin
NP 1	D-tyr^S	168 (Armstrong *et al.*, 1970; Burkholder and Giles, 1947)	Spontaneous reversion[b] (Kane *et al.*, 1971)
SS 101	D-tyr^R	NP 1	Spontaneous mutation
NP 93	D-tyr^R	BG 23 (Armstrong *et al.*, 1970; Burkholder and Giles, 1947)	Spontaneous reversion[b] (Champney and Jensen, 1969)
H-36	D-tyr^S	NP 93	Nitrosoguanidine treatment[c]

[a] The notations for D-tyrosine resistant or D-tyrosine-sensitive phenotypes are D-tyr^R or D-tyr^S, respectively.

[b] Parent strain 168 is an auxotroph which requires tryptophan; parent strain BG 23 requires threonine (Burkholder and Giles, 1947).

[c] Mutant H-36 was obtained by mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine as described under Methods. No selection was used, and survivors were screened by the replica plating technique for mutation to D-tyrosine sensitivity.

Table 2. The D-tyrosine-resistance phenotype of various *B. subtilis* isolates

D-tyrosine concentration	Specific growth rate (k)[a]			
	Strain 23		Strain 168	
μg per ml	NP 93	H-36	NP 1	SS 101
0	0.54	0.53	0.54	0.67
15	—	0.45	0.29	—
50	0.56	—	—	0.56

Inocula were grown in minimal salts-glucose medium at 37°C to an optical density at 600 nm of 0.600. The cells were centrifuged, resuspended at equal volume in minimal salts-glucose medium lacking a source of inorganic nitrogen and frozen overnight at liquid nitrogen temperature. These cells in 5 ml portions were thawed at 37°C, inoculated to an optical density at 600 nm of 0.100 by dilution with nitrogen-deficient medium, and incubated with vigorous shaking at 37°C for 75 min. At this time $(NH_4)_2SO_4$ at a final concentration of 0.2% and the indicated amount of D-tyrosine were added. Growth curves were obtained from turbidity data using a Klett-Summerson colorimeter (red filter).

[a] The specific growth rate, k (Herbert *et al.*, 1956), is a value proportional to the slope obtained in a growth curve; k was calculated as 0.69 divided by the doubling time in hours.

compositions of basic liquid media, stock slants, other solid media, and trace elements were given previously (Jensen, 1968).

Nitrosoguanidine Mutagenesis. N-methyl-N′-nitro-N-nitrosoguanidine (Aldrich Chemicals) was prepared in acetone at a concentration of 1 mg per ml. A 10 ml culture in exponential growth was centrifuged in a table top centrifuge, resuspended in 10 ml of minimal medium lacking glucose, and incubated at 37° C with shaking for 30 min following the addition of 0.05 ml of the stock solution of nitrosoguanidine. Survivors in the cell population were centrifuged, resuspended at equal volume in a trypticase soy-yeast extract medium (Jensen, 1968), and incubated at 37° C for an additional 2 h to allow segregation of mutant genomes.

Determination of Resistance to D-Tyrosine in Liquid Media. Growth rate determinations with D-tyrosine are apt to be quite variable in liquid medium. This is consistent with the progressive nature of the growth inhibition by D-tyrosine. It would appear that differences in inoculum carry-over and other aspects of culture history can cause very large differences during the relatively few generations of growth which are followed in the protocols of ordinary growth experiments. The procedure detailed under Table 2 was found to give reliable results in liquid growth determinations. Starvation of inocula cells for nitrogen apparently led to the depletion of the endogenous reserves of L-tyrosine, providing for a maximal and consistent inhibitory action of the D-isomer in growth experiments.

Uptake of D-Tyrosine in Whole Cells. Cultures of 10 ml volumes were grown in minimal-glucose medium to an optical density range at 600 *nm* of 0.400—0.500 (1 ml of culture having an absorbance at 600 nm of 1.0 corresponds to 450 μg dry weight of cell mass.) in 125-ml side-arm flasks. The exponentially-dividing cells were filtered, washed on the filter apparatus with 30 ml of nitrogen-deficient minimal salts-glucose medium and resuspended in 30 ml of the same medium for 60 min at 37° C with shaking. At this time culture turbidity was measured in Klett units. A 1.8 ml portion was withdrawn and added to 0.2 ml of D-tyrosine-1-^{14}C in a 13×100 mm tube at 37° C. The final concentration of D-tyrosine was 4.2 μM (0.2 μCi per ml). Also, $(NH_4)_2SO_4$ was added to a final concentration of 0.2%. Samples of 0.2 ml volume were withdrawn at carefully timed intervals, filtered through membrane filters with porosity of 0.45 μM, and washed with 10 ml volumes of minimal-glucose medium. The filters were dried under heat and placed in vials containing 10 ml volumes of Omnifluor (New England Nuclear). Due to the relatively slow rate of D-tyrosine assimilation, uptake was measured under conditions of growth in cells with depleted pools of L-tyrosine. Under these conditions, the incorporation of D-tyrosine was sufficient to yield reproducible data. The unit of uptake for D-tyrosine of 1 CPM per Klett unit corresponds to 0.01 μmoles of D-tyrosine per gram (dry weight) of cells.

Filtration Materials. Filter discs, 25 mm in diameter and 0.45 μM in pore size, were from Schl. & Sch., while 47 mm diameter discs (same porosity) were from Millipore. A Millipore vacuum-pressure pump was used in all filtration experiments.

Chemicals. D-tyrosine-1-^{14}C(47.6 μCi/μmole) and (U) ^{14}C-L-tyrosine (410 μCi/μmole) were purchased from New England Nuclear Co. Nonradioactive D-tyrosine (Calbiochem) was greater than 99.9% pure. Isotopic D-tyrosine contained less than 0.2% L-tyrosine. Other chemicals were of the finest reagent quality commercially available.

Results

Mutant derivatives of isolate NP 1 were selected for resistance to the growth inhibition ordinarily produced in the presence of D-tyrosine. Mutants were purified which did not cross-feed the sensitive background

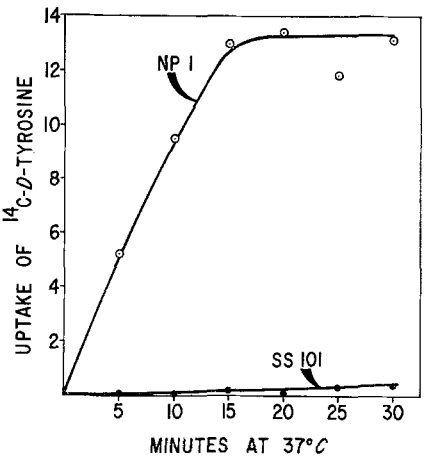

Fig. 1

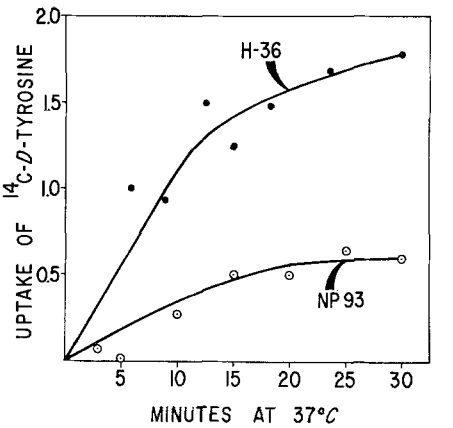

Fig. 2

Fig. 1. Comparison of uptake kinetics of D-tyrosine into whole cells of isolates NP 1 and SS 101. The incorporation of ^{14}C-D-tyrosine was as described under Materials and Methods. Uptake values on the ordinate scale are expressed as CPM per Klett unit of culture turbidity

Fig. 2. Comparison of uptake kinetics of D-tyrosine into whole cells of isolates NP 93 and H-36. The incorporation of ^{14}C-D-tyrosine was as described under Materials and Methods. Uptake values on the ordinate scale are expressed as CMP per Klett Unit of culture turbidity. Note difference in ordinate scale compared to Fig. 1

population on D-tyrosine-containing minimal agar plates [L-tyrosine excretors are regulatory mutants (Champney and Jensen, 1969)]. A representative mutant of this class, SS 101, showed no detectable cross-feeding of tyrosine auxotrophs on minimal salts-glucose agar or of wild type NP 1 on D-tyrosine-containing media. Furthermore, supernatant fluid collected from cultures of SS 101 did not inhibit the transport of ^{14}C-L-tyrosine by whole cells of isolate NP 1. The data in Fig. 1 clearly reveal the inability of mutant SS 101 to assimilate D-tyrosine into whole cells, in comparison to the whole cell incorporation of D-tyrosine observed in isolate NP 1. Hence, the basis of the resistance to growth inhibition by D-tyrosine in SS 101 is a mutational alteration in the system which transports D-tyrosine into the cell.

Like the mutant derivative of strain 168 (SS 101), the prototrophic isolate of strain 23 (NP 93) does not incorporate significant levels of D-tyrosine into whole cells (Fig. 2). On the other hand, a derivative of NP 93 (H-36) which possessed a mutation causing sensitivity to growth inhibition by D-tyrosine also displayed a membrane that was permeable to D-tyrosine.

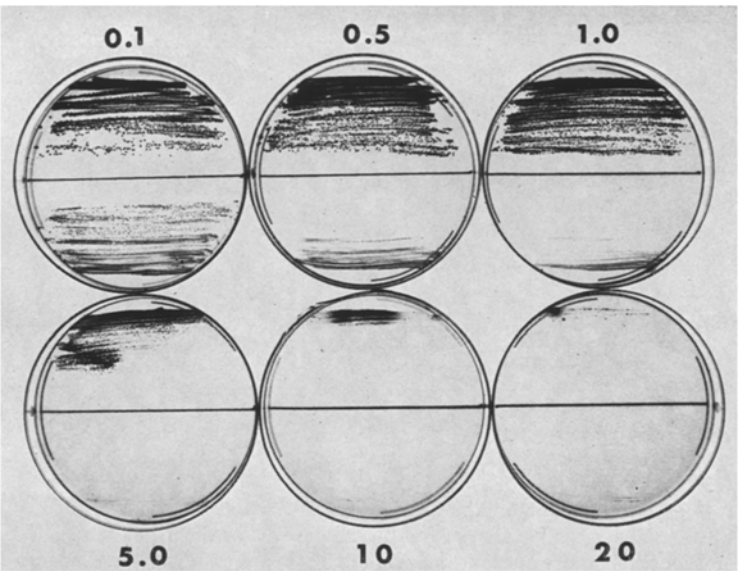

Fig. 3. Comparison of growth inhibition by D-tyrosine in isolates H-36 (top half of each agar plate) and NP 1 (bottom half of each agar plate). Agar petri plates containing minimal salts-glucose medium were prepared to contain the final concentrations (in μg per ml) of D-tyrosine indicated on the photographs. Cells of either NP 1 or H-36 from a single isolated colony on a 24 h BAB (Jensen, 1968) agar plate were transferred by means of a bacteriological loop and streaked continuously on one half of the plate. Plates were incubated for 48 h at 37° C before photography

Of the two D-tyrosine-permeable culture isolates used in these studies, isolate NP 1 incorporates relatively more D-tyrosine into whole cells than does mutant H-36. This correlates with the greater sensitivity of isolate NP 1 to growth inhibition by exogenous D-tyrosine (Table 2). Since the molecular mechanism of anti-metabolite action by D-tyrosine results in growth inhibition that is progressive, involving a steady increase in the intracellular ratio of D-tyrosine to L-tyrosine, differences between stains are amplified and more apparent after many generations of growth (Champney and Jensen, 1970a). Hence, the comparison of isolated colony formation on agar (shown in Fig. 3) is a more sensitive methodology than is turbidity measurement in liquid medium (Table 2).

Discussion

Although strains 168 and 23 can interchange genes by deoxyribonucleate-mediated transformation or by transduction, they differ in many respects (Armstrong *et al.*, 1970; Kane *et al.*, 1971). One striking

contrast between strains 168 and 23 of *B. subtilis* is that of sensitivity to growth inhibition caused by D-tyrosine (Champney and Jensen, 1969; Champney and Jensen, 1970a; Kane *et al.*, 1971). Derivatives of strain 168 are very susceptible to growth inhibition by D-tyrosine and may be considered unusual in this respect since few bacterial species are sensitive to growth inhibition by D-tyrosine. Previous enzymological results showed identical *in vitro* potential for false feedback inhibition of prephenate dehydrogenase by D-tyrosine in strains 23 and 168 (Champney and Jensen, 1970b). The following conclusion is consistent with the similar characteristics of prephenate dehydrogenase in the two strains of *B. subtilis*. Apparently, an intracellular potential exists for D-tyrosine inhibition of cellular growth in strain 23, once the permeability barrier is broken.

The gene for the "natural" D-tyrosine resistance of strain 23 was shown to have a different chromosomal location than the structural gene for prephenate dehydrogenase (Champney and Jensen, 1969). Hence, this regulatory protein could be eliminated as the basis of the strain difference. Other possible explanations for the resistance of strain 23 to D-tyrosine considered were (i) the possible presence of a D-tyrosine racemase, (ii) a D-tyrosyl-tRNA deacylase activity such as exists in *E. coli* (Calendar and Berg, 1967), (iii) a more specific tyrosyl tRNA activating enzyme, or (iv) impermeability to D-tyrosine. These experiments show at least the latter explanation to apply. It was possible to isolate mutants of strain 23 that are sensitive to growth inhibition by D-tyrosine as well as derivatives of strain 168 which are resistant to growth inhibition by D-tyrosine. In all cases sensitivity to growth inhibition by D-tyrosine correlated with D-tyrosine uptake while resistance to growth inhibition by D-tyrosine correlated with lack of D-tyrosine uptake. It seems likely that the natural D-tyrosine-resistance marker of strain 23 marks the chromosomal location of a gene concerned with tyrosine transport. It is assumed that this locus and the resistance locus of isolate SS 101 will locate together on the genetic map.

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