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Thymidylate Synthesis and Aminopterin Resistance in *Bacillus subtilis*

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ABSTRACT

WILSON, MELBA CARR (Brown University, Providence, R.I.), JAMES L. FARMER, AND FRANK ROTHMAN. Thymidylate synthesis and aminopterin resistance in *Bacillus subtilis*. J. Bacteriol. **92**:186–196. 1966.—The thymine-requirement of *Bacillus subtilis* 168 *thy* results from mutation in two unlinked genes (i.e., genetic loci) designated *thyA* and *thyB*. The *thyB* gene is located between the *met* and *ile* markers. Both *thyA*$^+$ *thyB* and *thyA* *thyB*$^+$ strains are phenotypically *thy*$^+$. *ThyA*$^+$ *thyB* strains resemble the wild type in their sensitivity to aminopterin, poor incorporation of exogenous thymine into deoxyribonucleic acid (DNA), and high level of thymidylate synthetase activity in crude extracts. *ThyA thyB*$^+$ strains are resistant to aminopterin in the presence of thymine, incorporate exogenous thymine into DNA, and have no detectable thymidylate synthetase activity. Experiments designed to elucidate the role of the *thyB* gene indicate that it specifies an alternate pathway of thymidylate synthesis, similar to thymidylate synthetase but requiring a cofactor other than tetrahydrofolate. The mechanism of selection of thymine-requiring mutants by aminopterin is revealed by these results.

The folic acid antagonist aminopterin indirectly prevents thymidylate biosynthesis by inhibition of dihydrofolate reductase, an essential enzyme in the synthesis of N^5,N^{10}-methylenetetrahydrofolate which is a precursor of the methyl group of thymidylate (Fig. 1). Aminopterin has been used in obtaining thymine-requiring (*thy*) mutants in several genera of bacteria, including *Escherichia coli* (23), *Salmonella typhimurium* (22), *Bacillus megaterium* (27), and *B. subtilis* (8). The *B. subtilis thy* mutant selected in this manner lacks the enzyme thymidylate synthetase.

Okada, Yanagisawa, and Ryan (24) showed that aminopterin acts as a selective agent for spontaneous thymine-requiring mutants, not as a mutagenic agent. They found the *thy* mutants to be altered in two phenotypic properties in addition to the thymine requirement: they are aminopterin resistant (*ami-r*) and are able to utilize exogenous thymine much more efficiently than the wild-type strains. Crawford (7) and Mantsavinos and Zamenhof (19) found that *thy*$^+$ revertants of *thy* mutants of *E. coli* retain the ability of the mutant strains to incorporate exogenous thymine. We have observed that some *thy*$^+$ transformants of *B. subtilis thy* mutants are aminopterin-resistant if grown in the presence of thymine. These results suggested to us that *thy* mutants arise from mutations at two genetic sites.

In this paper, we describe the genetic analysis of *B. subtilis* 168 *thy* mutants which shows that the *thy* phenotype results from mutations in two unlinked genes (i.e., genetic loci). Strains having the wild-type allele of either gene are *thy*$^+$. One of the genes (*thyA*) specifies thymidylate synthetase. The results described below indicate that the second gene (*thyB*) specifies a second, hitherto unknown, pathway for the synthesis of thymidylate.

MATERIALS AND METHODS

Bacterial strains. B. subtilis strains 23 (wild) and 168 *ind* were obtained from B. Strauss. Strain 168 *met ile leu* (Mu8u5u1) was obtained from N. Sueoka. The isolation of strain 168 *thy ind* was described previously by Farmer and Rothman (8). Strain 168 *met ile leu thyA2 thyB* was prepared by treating 168 *met ile leu thyA*$^+$ *thyB* with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.) and screening the surviving cells by replica

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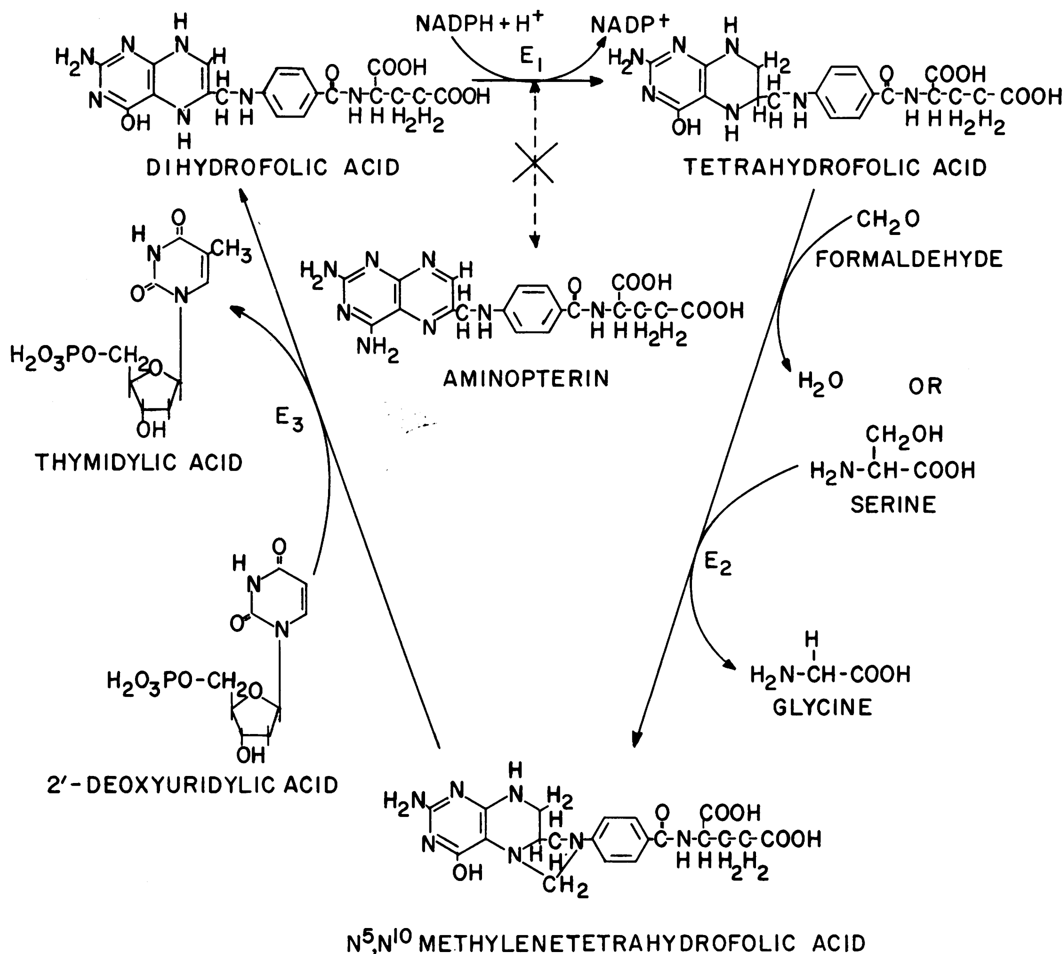

FIG. 1. *Relationship of aminopterin to thymidylate synthesis.* E_1 = *dihydrofolate reductase;* E_2 = L-*serine hydroxymethyl transferase;* E_3 = *thymidylate synthetase.*

plating. Derivation of each of the other strains used in this study is shown in Table 1.

Media. The broth medium used was Difco Antibiotic Medium No. 3. In addition, Spizizen's minimal salts (1) plus 0.5% glucose (SG), SG plus 1% monosodium glutamate (SGG), and Difco Tryptose Blood Agar Base plus 20 μg/ml of thymine were used. Supplements (L-isoleucine, L-leucine, L-methionine, and thymine) were normally added to a final concentration of 20 μg/ml.

Isotopes. Thymine-2-C^{14} and C^{14}(-methyl)-methionine were purchased from New England Nuclear Corp., Boston, Mass.

Antibiotics. Aminopterin was purchased from Calbiochem. 5-Fluoro-2'-deoxyuridine was a gift from Hoffman-La Roche, Inc., Nutley, N.J.

Transformation. Transforming DNA was prepared by a modified version of the Marmur (20) technique in which the steps following the first ethyl alcohol precipitation were omitted. Competent cells were prepared as described by Anagnostopoulos and Spizizen (1) with the modification described previously (8). Deoxyribonucleic acid (DNA) concentrations were determined by the diphenylamine reaction of Dische (2). Nonsaturating concentrations of DNA were used in the crosses described in this paper.

Plating. Up to one-third of *thy*+ transformants die if placed directly onto selective medium (Farmer, Ph.D. Thesis, Brown Univ., Providence, R.I., 1966). A technique based on one described by Iyer (15) was developed to prevent this loss in experiments where quantitation of results was important. Transformed cells were transferred to black filters (Millipore HABP 047), and the filters were placed on complete agar medium. After 3 to 4 hr at 37 C, the filters were transferred first to selective soft-agar medium for 30 min, and then to selective agar medium. The "blotting" on soft agar was absolutely necessary to deplete the supplements which were retained in the filter.

Preparation of exponentially growing cultures. Cells freshly grown on Difco Tryptose Blood Agar Base plus 20 μg/ml of thymine were lightly suspended in

the desired medium, serially diluted, and grown overnight. The culture with optical density at 540 mμ (OD_{540}) between 0.1 and 1.0 (10^7 to 10^8 cells per milliliter) was used as the inoculum for the day's experiment.

Incorporation of C^{14}-thymine. Exponentially growing cells were diluted into medium containing 5 μg/ml of C^{14}-thymine with or without 200 μg/ml of aminopterin, and were aerated by bubbling at 37 C. At intervals, the OD_{540} was measured, and a sample was assayed for 5% trichloroacetic acid-precipitable radioactive material.

Thymidylate synthetase assays. Crude cell extracts in 0.05 M tris(hydroxymethyl)aminomethane plus 0.01 M mercaptoethanol plus 0.001 M ethylenediaminetetraacetate (*p*H 7.4) were prepared by grinding cells with alumina and were clarified by centrifugation at 100,000 × *g* for 1 hr. Assays were performed as described by Friedkin (11). Protein concentration was determined by the method of Lowry et al. (18). (Since mercaptoethanol gives a positive reaction with the Folin reagent, determinations were carried out on trichloroacetic acid precipitates of the extracts.)

DNA hydrolysis. DNA prepared by the method of Saito and Miura (25) was dissolved in 70% perchloric acid and heated at 100 C for 2 hr. The resulting black residue was extracted with water and chromatographed on Whatman no. 40 paper in each of two solvents: (I) 680 ml of isopropanol, 176 ml of concentrated HCl, diluted to 1,000 ml with water; (II) 860 ml of *n*-butanol, 140 ml of water, in a chamber saturated with ammonia.

Results and Discussion

Linkage of thy to the methionine-isoleucine linkage group. The results of cross 1 (Table 2) show that the *thy* gene is in the methionine-isoleucine linkage group (29). Of 211 *thy*$^+$ transformants, 62 (30%) also carried the donor allele for *met*, *ile*, or both. However, in the reciprocal cross for these markers in which the donor DNA was prepared from the *thy* strain, no linkage was observed between the thymine and the methionine and isoleucine genes, although linkage of the latter two was not disrupted (cross 2, Table 3).

This unexpected result may be explained by postulating that the *thy* strain contains mutations at two unlinked genetic loci, *thyA* and *thyB*, only one of which (*thyB*) is in the methionine-isoleucine linkage group, and that either *thyA*$^+$ or *thyB*$^+$ alone confers the *thy*$^+$ phenotype. If this is the case, we may note the genotypes of the parental strains in crosses 1 and 2 as *met ile thyA*$^+$ *thyB*$^+$ and *met*$^+$ *ile*$^+$ *thyA thyB*. Since *met*$^+$, *ile*$^+$, and *met*$^+$ *ile*$^+$ transformants in cross 2 would retain the *thyA*$^+$ marker of the recipient cells, they would be phenotypically *thy*$^+$, even if they carried the *thyB* marker.

To test the hypothesis of two unlinked *thy* genes, we transformed a *thy*$^+$ recombinant strain obtained from cross 1 (presumptive genotype *met ile thyA thyB*$^+$) with DNA from two *met*$^+$ *ile*$^+$ transformants isolated from cross 2 (presumptive genotype *met*$^+$ *ile*$^+$ *thyA*$^+$ *thyB*), and scored the frequency of *met*$^+$ *ile*$^+$ *thy*$^+$ and *met*$^+$ *ile*$^+$ transformants. Although both parents in each cross were phenotypically *thy*$^+$, only 17% of the *met*$^+$ *ile*$^+$ transformants had the *thy*$^+$ phenotype (Table 4). This result was subsequently confirmed in a similar cross in which 171 of 213 (80%) *met*$^+$ *ile*$^+$ transformants selected on thymine-supplemented plates were found to be *thy*.

Mapping of the thyB gene. To determine the degree of linkage among the *met*, *ile*, *and thyB* genes, strain *met ile thyA thyB* was transformed with *met*$^+$ *ile*$^+$ *thyA*$^+$ *thyB* DNA. The number of single and double transformants of each pheno-

TABLE 1. *Origin of strains of Bacillus subtilis used*

DNA donor	Recipient	Selected markers	Derivative
168 *thy ind*	168 *met ile leu*	*ami-r*[a]	168 *met ile leu thyA thyB*$^+$
168 *thy ind*	168 *met ile leu*	*met*$^+$ *ile*$^+$	168 *leu thyA*$^+$ *thyB*
23 (wild)	168 *thy ind*	*ind*$^+$	168 *thy*
168 *met ile leu*	168 *thy*	*thy*$^+$	168 *met ile thyA thyB*$^+$
168 *thy ind*	168 *met ile thyA thyB*$^+$	*met*$^+$	168 *ile thy*
168 *ile thy*	168 *met ile leu thyA thyB*$^+$	None[b]	168 *met ile leu thy*
168 *ind*	168 *met ile leu thy*	*met*$^+$ *ile*$^+$	168 *leu thyA thyB*$^+$
168 *leu thyA*$^+$ *thyB*	168 *met ile leu thy*	*thy*$^+$	168 *met ile leu thyA*$^+$ *thyB*

[a] In presence of thymine. Less than 5% of the colonies which grew on plates containing aminopterin, L-methionine, L-isoleucine, L-leucine, and thymine were of this genotype. The others, which were smaller, were resistant to aminopterin in the absence of thymine also. We conclude that strain 168 *thy ind* contains this mutation to "absolute" aminopterin resistance in addition to the *thyA* mutation which confers resistance only in the presence of thymine.

[b] *Thy* transformants were detected by their transparent, granular appearance on plates containing SG medium plus L-methionine, L-isoleucine, L-leucine, and thymine. *Thy*$^+$ colonies are opaque, nongranular.

type, as well as the number of triple transformants, was determined by scoring the number of colonies on various selective media (Table 5). The extent of linkage between any two markers may be quantitatively expressed as the cotransfer index, which is defined as the number of double transformants divided by the sum of the two single transformant types plus the double transformants (21). We may illustrate the calculation of the cotransfer indices listed in Table 5 for the *met* and *ile* markers. The cotransfer index is by definition:

$$\frac{met^+\ ile^+}{met\ ^+ile + met\ ile^+ + met^+\ ile^+}.$$

The number of *met*⁺ *ile*⁺ transformants is obtained directly from the count on L-leucine–thymine plates. Since the transformants scored as *met*⁺ (L-isoleucine–L-leucine–thymine plates) and as *ile*⁺ (L-methionine–L-leucine–thymine plates) each include the *met*⁺ *ile*⁺ double transformants, the cotransfer index is equal to:

$$\frac{met^+\ ile^+}{met^+ + ile^+ - met^+\ ile^+}$$

$$= \frac{1.8 \times 10^3}{6.8 \times 10^3 + 4.7 \times 10^3 - 1.8 \times 10^3} = 0.18$$

The results show that *ile* and *thy* are closer to each other than to *met*.

The accuracy of cotransfer indices calculated from direct transformant counts depends on the absence of any selective survival among the various transformant classes. In control experiments, Farmer (Ph.D. Thesis, Brown Univ., Providence, R. I., 1966) found that up to one-third of the potential thy^+ transformants may undergo thymineless death if plated directly after transformation on plates lacking thymine. This death can be avoided by preincubation of the transformants (collected on Millipore membranes) on thymine-supplemented plates for 3 to 4 hr prior to transfer to selective plates. The preincubation method was used in the cross shown in Table 5. Results of similar crosses in which the transformants were plated directly gave similar values for the cotransfer indices: *met-thyB*, .20–.23; *ile-thyB*, .53–.-56 *met-ile*, .20–.22 (Wilson, Ph.D. Thesis, Brown Univ., Providence, R.I., 1966).The *met-thy* and *ile-thy* cotransfer indices are not expected to be very sensitive to death of a fraction of the thy^+ transformants, provided a similar fraction of the double transformants also die.

The order of the *met*, *thyB*, and *ile* genes could not be unambiguously determined from the cotransfer indices because of the proximity of the *ile* and *thyB* genes. The order was determined, however, by the three-factor crosses illustrated in Table 6. In each cross, ile^+ thy^+ transformants were selected by use of the preincubation technique and were scored for the *met* marker. These crosses established the order as *ile thyB met*.

The absence of linkage between the *thyA* gene and the *met-ile* linkage group was confirmed by the cross shown in Table 7.

Growth in the presence of aminopterin. Strains of genotype $thyA^+$ *thyB*, like the wild type, do not grow on plates containing aminopterin or aminopterin plus thymine. Strains which are *thyA*

TABLE 2. *Linkage of thy⁺ to met and ile*[a]

DNA concn (μg/ml)	Primary selection	No. of recombinants in selected class				
		met^+ ile^+ leu^+	met ile^+ leu^+	met^+ ile leu^+	met^+ ile^+ leu	met ile leu^+
0.025	thy^+	57	3	8	0	2
0.05	thy^+	40	4	18	0	8
0.5	thy^+	52	4	11	0	4

[a] Cross 1: 168 *met ile leu* (DNA donor) × 168 *thy* (recipient).

TABLE 3. *Absence of linkage of thy to met and ile*[a]

Primary selection	No. of recombinants			
met^+	19 *ile* thy^+	17 ile^+ thy^+	0 *ile thy*	0 ile^+ *thy*
ile^+	27 *met* thy^+	8 met^+ thy^+	0 *met thy*	0 met^+ *thy*
met^+ ile^+	—	36 thy^+	0 *thy*	—

[a] Cross 2: 168 *thy ind* (DNA donor) × 168 *met ile leu* (recipient).

TABLE 4. *Confirmation of the existence of two unlinked thy gene loci*[a]

DNA isolate[b]	Transformants/ml		met^+ ile^+ thy^+ / met^+ ile^+
	met^+ ile^+ thy^+	met^+ ile^+	
1	5.0×10^2	2.8×10^3	0.18
2	8.5×10^2	5.0×10^3	0.17

[a] Cross: 168 *leu* $thyA^+$ *thyB* (DNA donor) × 168 *met ile thyA* $thyB^+$ (recipient).
[b] DNA was prepared from two *leu* $thyA^+$ *thyB* transformants from cross 2.

TABLE 5. *Frequency of cotransformation of met, ile, and thyB*[a]

Supplements in plates[b]	Selected marker(s)	Colonies per plate	Dilution	Transformants[c]/ml
ILT	*met*+	349, 321, 346	20 ×	6.8×10^3
MLT	*ile*+	256, 235, 213	20 ×	4.7×10^3
MIL	*thy*+	242, 235, 226	20 ×	4.7×10^3
IL	*met*+ *thy*+	85, 83, 100	20 ×	1.8×10^3
ML	*ile*+ *thy*+	167, 152, 170	20 ×	3.3×10^3
LT	*met*+ *ile*+	102, 81, 84	20 ×	1.8×10^3
L	*met*+ *ile*+ *thy*+	76, 69, 61	20 ×	1.4×10^3

[a] Cross: 168 *leu thyA thyB*+ (DNA donor) × 168 *met ile leu thyA thyB*. Cotransfer indices: *met-thyB*, 0.18; *ile-thyB*, 0.54; *met-ile*, 0.18.
[b] I = L-isoleucine; L = L-leucine; m = L-methionine; T = thymine.
[c] Control plating of the recipient cells showed less than 20 revertants per ml for all markers.

TABLE 6. *Three-factor crosses to order met, ile, thy*[a]

Cross	DNA donor	Recipient	No. of *ile*+ *thy*+ colonies picked	No. of recombinants	
				met+ *ile*+ *thy*+	*met ile*+ *thy*+
3	*ile*+ *thyB met*+	*ile thyB*+ *met*	264	31 (12%)	233 (88%)
4	*ile thyB*+ *met*	*ile*+ *thyB met*+	280	133 (48%)	147 (52%)

[a] Plating method: preincubation.

thyB+, like *thy* mutants, do grow on plates containing aminopterin plus thymine but do not grow if thymine is omitted. We shall refer to strains of the latter type as being conditionally resistant to aminopterin. (See the first footnote to Table 1 for properties of strain 168 *ind thy*.)

The effect of aminopterin on the growth of the three phenotypically *thy*+ genotypes in the presence and absence of exogenous thymine was further studied in liquid culture (Fig. 2). As measured by increase of optical density of the culture and viable-cell count, growth of the wild-type and *thyA*+ *thyB* strains continued for a short time before leveling off, both in the presence and in the absence of thymine. Different results were obtained with strain *thyA thyB*+. When grown in aminopterin without thymine, the OD increased about 20-fold and then decreased, and the viable count decreased sharply, after a small increase, in a manner characteristic of thymineless death. When grown in aminopterin with thymine, this conditionally resistant *thyA thyB*+ strain continued to grow for at least 29 doublings with a doubling time 1.7 times that in the absence of aminopterin. Aminopterin had no effect on the growth rate of strain *thyA thyB* for at least 25 doublings.

Uptake of thymine-2-C[14]. Uptake of thymine-2-C[14] by the four strains in the presence and absence of aminopterin is shown in Table 8. Strains which are *thyA* incorporated much larger amounts of exogenous thymine than did strains which are *thyA*+. Adding aminopterin during the labeling period increased the amount of thymine incorporated into all three *thy*+ strains.

TABLE 7. *Absence of linkage of thyA to met and ile*[a]

Supplements in plates[b]	Selected marker(s)	Colonies per plate	Dilution	Transformants[c]/ml
ILT	*met*+	148, 183	50 ×	8.3×10^3
MLT	*ile*+	229, 229	50 ×	1.1×10^4
MIL	*thy*+	210, 217	50 ×	1.3×10^4
IL	*met*+ *thy*+	1, 0	10 ×	5
ML	*ile*+ *thy*+	0, 0	10 ×	0
LT	*met*+ *ile*+	248, 223	10 ×	2.4×10^3
L	*met*+ *ile*+ *thy*+	1, 0	10 ×	5

[a] Cross: 168 *leu thyA*+ *thyB* (DNA donor) × 168 *met ile leu thyA thyB* (recipient). Cotransfer indices: *met-thyA*, <0.001; *ile-thyA*, <0.001; *met-ile*, 0.14. Transformants were plated directly on selective media without preincubation.
[b] I = L-isoleucine; L = L-leucine; M = L-methionine; T = thymine.
[c] Control plating of the recipient cells per 0.1 ml: 3 *met*+, 1 *thy*+, and 0 for all others.

Thymidylate synthetase assays. Thymidylate synthetase assays of crude extracts of cells of the four *thy* genotypes (Table 9) demonstrated that activity is lost in *thyA* mutants but is unaffected by mutation in the *thyB* gene.

Bodmer and Grether (4) reported that uracil supplied in the growth medium of *B. subtilis* is not

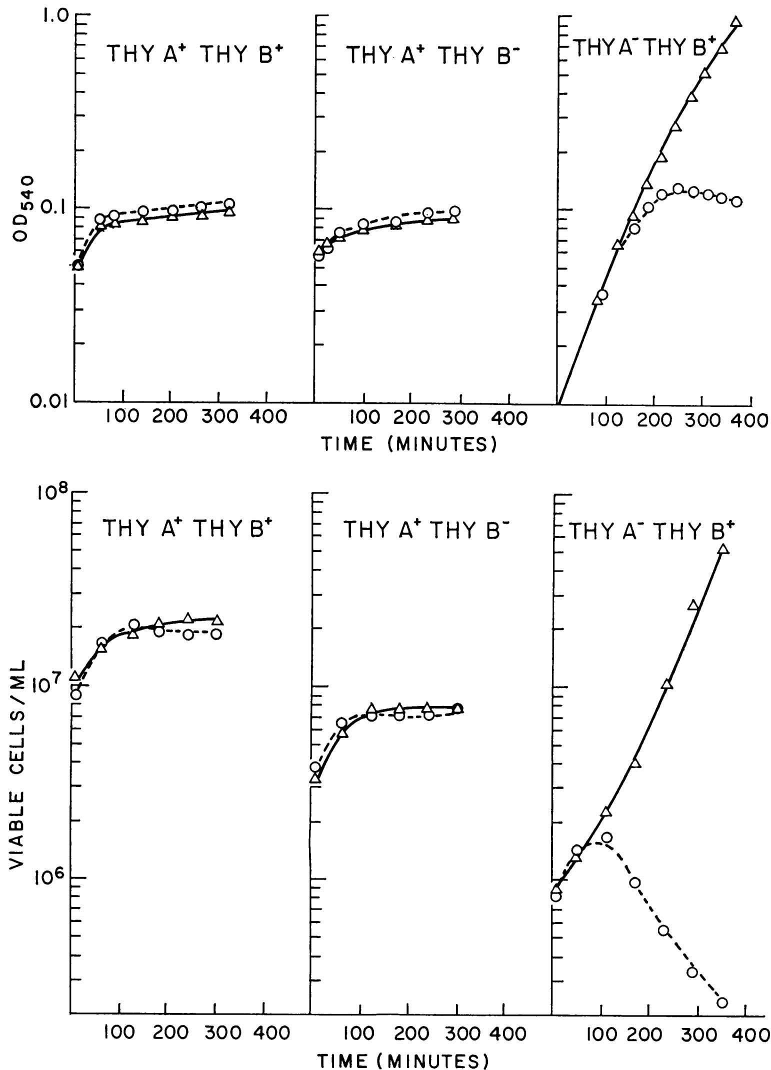

FIG. 2. *Growth of 168 met ile leu thyA$^+$ thyB$^+$, 168 leu thyA$^+$ thyB, and 168 met ile leu thyA thyB$^+$. Exponentially growing cells of the three strains in SGG plus L-methionine, L-isoleucine, and L-leucine were diluted into SGG plus L-methionine, L-isoleucine, and L-leucine, plus 200 μg/ml of aminopterin (○) and the same medium supplemented with 20 μg/ml of thymine (△) at time zero, and aerated by bubbling at 37 C.*

incorporated into DNA. The demonstration that deoxyuridylate is a precursor of thymidylate (F. and E. S. Kahan, *personal communication*; Table 9) indicates that the conversion of uracil to deoxyuridylate does not take place under the conditions used by Bodmer and Grether.

Summary of phenotypic properties. Table 10 summarizes the properties of strains having the four *thy* genotypes. Conditional aminopterin resistance, ability to incorporate exogenous thymine, and loss of thymidylate synthetase activity all result from mutation in the *thyA* gene. The properties of strain *thyA thyB$^+$* are most unexpected: it is phenotypically *thy$^+$*, but has no detectable thymidylate synthetase activity.

We have considered three possible explanations for the properties of strain *thyA thyB$^+$* and the role of the *thyB* gene: (i) Strain *thyA thyB$^+$* has an unusual base replacing thymine in its DNA. This possibility was suggested by the findings that some *B. subtilis* bacteriophage contain uracil or hydroxymethyluracil in place of thymine in their DNA (17, 26). By this hypothesis, the *thyB* gene would control an essential step in the synthesis of the unusual nucleotide. (ii) The *thyB* gene is a suppressor gene which restores activity to the

thyA gene product. (iii) The *thyB* gene corresponds to an alternate pathway for the synthesis of thymidylate.

Experiments designed to distinguish among these alternatives are described in the following sections.

Base analysis of DNA from strains thyA thyB$^+$ *and thyA*$^+$ *thyB*$^+$. Hydrolysates of DNA from the two strains were chromatographed on paper (Table 11). The hydrolysates behaved identically, each containing a spot corresponding exactly with the thymine standard. Uracil was well separated from thymine in solvent II, whereas hydroxymethyl uracil would have been destroyed under the hydrolysis conditions employed. The adenine and presumptive thymine spots which were developed in solvent I were eluted with 0.1 N HCl. The ultraviolet spectra (in acid) from 220 to 290 mμ of the presumptive thymine spots of the two strains were identical to each other and to the spectrum of the eluted thymine standard. The adenine-thymine ratios calculated from the extinction coefficients were approximately equal (1.3 for the control and 1.2 for *thyA thyB*$^+$). On the basis of this experiment, we conclude that the *thyA thyB*$^+$ strain contains the usual amount of thymine in its DNA.

TABLE 8. *Incorporation of* C^{14}*-thymine*

Strain	Thymine incorporated (mμmoles) per cell per doubling	
	Without aminopterin	With aminopterin
168 *met ile leu*[a]	0.005×10^{-8}	0.5×10^{-8}
168 *leu thyA*$^+$ *thyB*[a]	0.005×10^{-8}	0.1×10^{-8}
168 *met ile leu thyA thyB*$^+$[a]	0.08×10^{-8}	0.2×10^{-8}
168 *thy ind*[b]	1.7×10^{-8}	—

[a] Grown in SGG plus L-methionine, L-isoleucine, and L-leucine (plus 5 μg/ml of thymine during labeling period).

[b] Grown in SGG plus 20 μg/ml of tryptophan plus 5 μg/ml of thymine.

Is thyB a suppressor gene for thyA? If *thyB* is a suppressor of *thyA*, and restores thymidylate synthetase activity in vivo, it has to function in such a way that the "suppressed" enzyme is too labile to be assayed in crude extracts. Moreover, we have not been able to account for the behavior of strain *thyA thyB*$^+$ when exposed to aminopterin by any suppressor gene model.

Since suppressor mutations are generally site-specific, we examined the effect of the *thyB*$^+$ gene on an independently isolated *thyA* mutant. The *thyA2* mutation of strain 168 *met ile leu thyA2 thyB* was isolated without the use of aminopterin to guard against the possibility that aminopterin selects for a particular class of *thyA* mutants. This strain was transformed with DNA from wild-type *B. subtilis* 23. *Met*$^+$ *ile*$^+$ transformants, 90% of which received the *thyB*$^+$ gene, were tested for thymine phenotype and aminopterin resistance.

TABLE 9. *Thymidylate synthetase assays of crude extracts*[a]

Genotype	Medium in which cells were grown[b]	Millimicromoles of thymidylate formed/hr/mg protein at 20 C
168 *met ile leu thyA*$^+$ *thyB*$^+$	Broth	43
	SGG + MILT	29
	SGG + MIL	34
	SGG + MIL	41
168 *leu thyA*$^+$ *thyB*	Broth	38
168 *met ile thyA thyB*$^+$	Broth	<3.5
168 *met ile leu thyA thyB*$^+$	Broth	0.0
	SGG + MILT	<1.7
	SGG + MIL	<0.88
	SGG + MIL	<0.36
168 *ile leu thyA thyB*	Broth	<0.98

[a] The reaction mixture contained deoxyuridylate (0.05 μmole), d,l L-tetrahydrofolate (0.33 μmole), formaldehyde (15 μmole), $MgCl_2$ (25 μmole), mercaptoethanol (130 μmole), and 0.9 ml of crude extract in a final volume of 1.2 ml, at pH 7.4. The change in OD at 340 mμ was followed against a reference cuvette which contained the same reaction mixture except that deoxyuridylate was omitted. The absorption change measures the oxidation of tetrahydrofolate to dihydrofolate (11).

[b] Broth = Difco Antibiotic Medium No. 3; SGG = Spizizen's minimal salts (1) plus 0.5% glucose and 1% monosodium glutamate; M = L-methionine; I = L-isoleucine; L = L-leucine; T = thymine.

TABLE 10. *Summary of properties of strains having the four thy genotypes*

Genotype	Thymine phenotype	Thymidylate synthetase activity	Response to aminopterin in presence of thymine	Ability to incorporate exogenous thymine
thyA$^+$ *thyB*$^+$	*thy*$^+$	+	Sensitive	−
thyA$^+$ *thyB*	*thy*$^+$	+	Sensitive	−
thyA thyB$^+$	*thy*$^+$	−	Resistant	+
thyA thyB	*thy*	−	Resistant	+

TABLE 11. *Base analysis of DNA from strains 168 met ile leu thyA thyB*$^+$ *and 168 met ile leu thyA*$^+$ *thyB*$^+$ *(control)*

Compound	R_F	
	Solvent I	Solvent II
Thymine standard	0.78	0.45
Uracil standard	—	0.24
Cytosine standard	0.46	—
Adenine standard	0.32	—
Guanine standard	0.22	—
Thymine spot, control strain	0.76	—
Thymine spot, *thyA thyB*$^+$ strain	0.77	0.45

Of 50 *met*$^+$ *ile*$^+$ recombinants tested, 45 were phenotypically *thy*$^+$, indicating that all of the *thyA2 thyB*$^+$ cells were phenotypically *thy*$^+$. Of the 45 *thy*$^+$ recombinants tested, 44 were also conditionally aminopterin-resistant, indicating that the *thyA2* allele confers this phenotypic property as well. (The one sensitive recombinant presumably received the *thyA*$^+$ gene during the transformation.) This result supports the arguments *against* the suppressor gene explanation.

Alternate pathway of thymidylate synthesis. The results described thus far indicate that the most probable function of the *thyB* gene is specification of an enzyme in an alternate pathway for the synthesis of thymidylate. The unique step in thymidylate biosynthesis is the introduction of the methyl group at position 5 of the pyrimidine ring of deoxyuridylate (dUMP). Transfer of one-carbon units generally involves either a tetrahydropteroylglutamate cofactor or *S*-adenosylmethionine. Aminopterin is known to eventually inhibit the former type of reaction by blocking the production of tetrahydropteroylglutamates. The conditional resistance of strain *thyA thyB*$^+$ indicates that aminopterin inhibits the alternate pathway of thymidylate synthesis without blocking other essential one-carbon transfer reactions, making involvement of *S*-adenosylmethionine unlikely.

The following experiment supports this conclusion. A culture of *met ile leu thyA thyB*$^+$ was labeled with C^{14}-(-methyl)-methionine. Adenine and guanine were added to the growth medium to suppress incorporation of label into purines in DNA. DNA was isolated and purified by the method of Saito and Miura (25), and counted. The sample contained 1.5 counts per min per μg of DNA (28 counts per min in a 19-μg sample) compared with a theoretical value of 150 counts per min per μg for DNA whose thymine methyl groups had arisen entirely from methionine. This result eliminates pathways using *S*-adenosylmethionine, particularly the reduction of ribothymidylic acid, whose methyl group originates from reaction of *S*-adenosylmethionine with uridylic acid residues in ribonucleic acid (9, 12).

To investigate further the nature of the reaction determined by the *thyB*$^+$ gene, we examined the effect of 5-fluorodeoxyuridine (FUdR) on the growth of *thyA thyB*$^+$ cells. This compound is converted to the corresponding 5′ -nucleotide which is a potent inhibitor of thymidylate synthetase (6). Uracil was added to the culture to prevent incorporation into ribonucleic acid of any 5-fluorouracil produced. If dUMP were a substrate of the second pathway, FUdR should inhibit the growth of *thyA thyB*$^+$ cells and lead to thymineless death. Figure 3 shows that FUdR did lead to thymineless death of strain *thyA thyB*$^+$.

DISCUSSION

The genetic and physiological studies described above indicate that in *B. subtilis* there is a second pathway of thymidylate biosynthesis in addition to the conventional reaction catalyzed by thymidylate synthetase. A definitive knowledge of the reaction(s) involved must await the results of in vitro biochemical studies, which have been initiated. However, the results of the preceding sections allow us to draw certain conclusions about the nature of the reaction, and to propose a model which accounts for all of the data.

The addition of aminopterin to strain *thyA*$^+$ *thyB* leads to cessation of growth without thymineless death. This behavior indicates that not only thymidylate but precursors of protein or ribonucleic acid synthesis are also depleted (5). This result is expected since aminopterin inhibits new production of tetrahydrofolate (THFA), and the pool of THFA is rapidly depleted by reoxidation to dihydrofolate in the course of thymidylate synthesis catalyzed by thymidylate synthetase (Fig. 1), the product of the *thyA*$^+$ gene. THFA is a required cofactor in many reactions beside thymidylate synthetase, including biosynthesis of protein and ribonucleic acid precursors (10).

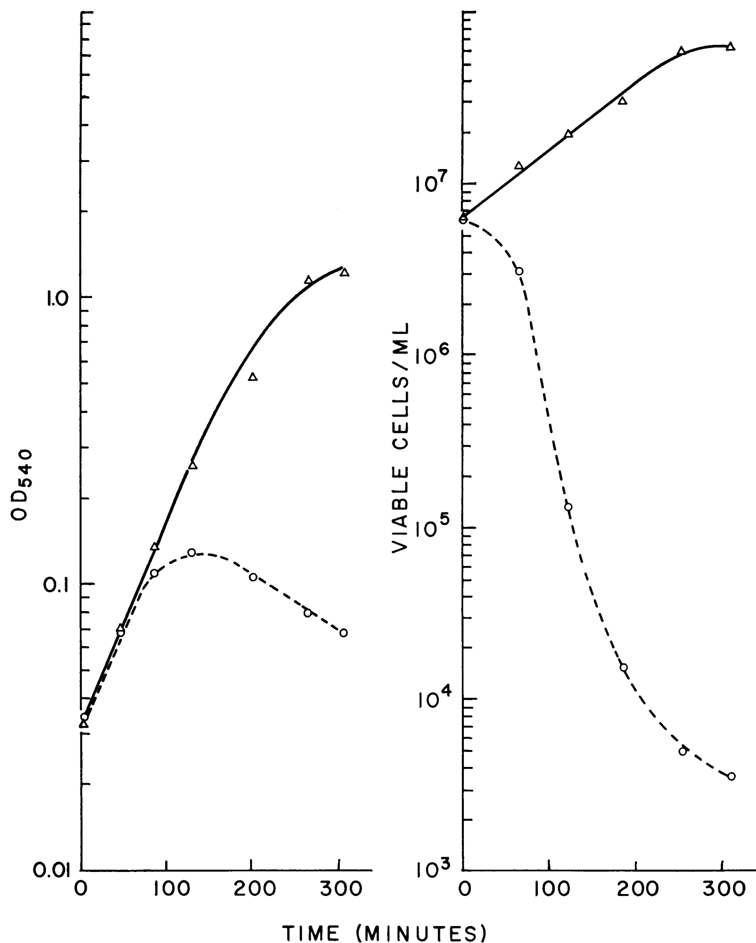

FIG. 3. *Response of 168 met ile leu thyA thyB⁺ to FUdR. Exponentially growing cells in SGG plus L-methionine, L-isoleucine, and L-leucine were diluted into SGG plus L-methionine, L-isoleucine, and L-leucine, plus 20 μg/ml of uracil and 20 μg/ml FUdR* (○) *and the same medium plus 20 μg/ml thymidine* (△) *at time zero, and were aerated by bubbling at 37 C.*

When strain *thyA thyB*$^+$ is grown without thymine, the only source of thymidylate is via the alternate pathway specified by the *thyB*$^+$ gene. Addition of aminopterin leads to cessation of growth *and to thymineless death* after about four generations. The cessation of growth indicates that the alternate pathway involves a compound whose synthesis is inhibited by aminopterin. We interpret the occurrence of thymineless death to mean that depletion of this compound leads to selective depletion of thymidylate and interruption of DNA synthesis. The fact that thymine alone overcomes the growth inhibition of aminopterin conclusively confirms this interpretation. Therefore, the "aminopterin-sensitive" compound is not required in any other reaction and cannot be THFA. We conclude that the alternate pathway requires a cofactor related to but not identical to THFA.

We believe that one of the tetrahydropteroylpolyglutamates, probably tetrahydropteroyltriglutamate ($THPtG_3$) is the most likely candidate as this cofactor for the following reasons: (i) The synthesis of $THPtG_3$ is catalyzed by dihydrofolate reductase (3, 30), and is therefore aminopterin-sensitive. (ii) $THPtG_3$ has been shown to be involved as a cofactor in one-carbon transfer reactions (16, 28). (iii) Pteroyltriglutamate and its 5-formyl derivative have been isolated from extracts of *B. subtilis* (14). (iv) Friedkin (*personal communication*) has also shown that the N^5,N^{10}-methylene derivative of $THPtG_3$ can substitute for the corresponding THFA derivative as cofactor for *E. coli* thymidylate synthetase.

The in vivo inhibition of the alternate pathway by FUdR (Fig. 3) suggests that dUMP is the substrate methylated in the alternate pathway, though more complicated alternatives involving deoxyuridine diphosphate or deoxycytidine derivatives are not rigorously excluded. We favor the simplest model, in which the *thyB* gene-product is an enzyme (enzyme B) which catalyzes the methylation of dUMP by a reaction similar to that catalyzed by thymidylate synthetase (Fig. 1), except that, instead of N^5,N^{10}-methyleneTHFA, another cofactor, e.g., N^5,N^{10}-methyleneTHPtG$_3$ is required. A closely analogous precedent exists for this model: there are two enzymes in *E. coli* which catalyze the synthesis of methionine by transfer of a methyl group to homocysteine. One of the enzymes can use either N^5-methylTHFA or N^5-methylTHPtG$_3$ as the methyl donor, whereas the other enzyme requires the latter (13).

This model accounts for the details of the response of strain *thyA thyB*$^+$ in the following manner. (For simplicity, we shall refer to the unknown cofactor as THPtG$_3$. The argument holds equally for any cofactor other than THFA whose synthesis is inhibited by aminopterin, and which is stoichiometrically used in the thymidylate-synthesizing reaction.) During growth in the presence of aminopterin without thymine, THPtG$_3$ is exhausted by oxidation to the dihydro form owing to thymidylate synthesis. *In other one-carbon transfer reactions, the tetrahydropterolyglutamate cofactors are not oxidized.* Since the conventional thymidylate synthetase is not active in this strain, THFA is not rapidly depleted under these conditions, other one-carbon transfers are not affected, and the selective interruption of thymidylate synthesis leads to thymineless death. The continued growth of strain *thyA thyB*$^+$ for unlimited periods in the presence of aminopterin and thymine indicates that the aminopterin block of dihydrofolate reductase is sufficiently "leaky" to permit the synthesis of the relatively small amount of THFA needed in the absence of thymidylate synthetase. This "leakiness" does not provide a sufficient supply of THFA in strain *thyA*$^+$ *thyB* because of the reoxidation of THFA to dihydrofolate during the thymidylate synthetase reaction. The fact that the reversal of the effect of aminopterin on strain *thyA thyB*$^+$ by thymine is not complete, but leads to exponential growth with a longer doubling time, indicates that THPtG$_3$ is an alternate though not required cofactor in some reaction(s) other than thymidylate synthesis.

Mutation of the wild-type strain to *thyA thyB*$^+$ results in three phenotypic changes: loss of thymidylate synthetase activity, acquiring of aminopterin resistance, and increased uptake of thymine by the cells. Mutation to *thyA*$^+$ *thyB* leads to none of these changes. How are these effects related? Although definite evidence is lacking, a reasonable explanation can be formulated by making the following *ad hoc* assumption: thymidylate synthetase is considerably more active than the alternate pathway; in *thyA*$^+$ strains, this activity results in a sufficiently large pool of thymidylate (or thymidine di- or triphosphate) to prevent conversion of thymine to thymidylate by inhibition or repression, or both, of the enzyme(s) catalyzing this conversion.

In strain *thyA*$^+$ *thyB*, thymidylate synthetase is functional, and the pool of thymidylate (or derivatives) prevents uptake of thymine. The strain is aminopterin-sensitive because depletion of THFA prevents the synthesis of many required compounds. This sensitivity is not overcome by thymine, even though the uptake of exogenous thymine is considerably increased in the presence of aminopterin (Table 8). (This increase is consistent with our *ad hoc* assumption.)

In strain *thyA thyB*$^+$, thymidylate synthetase is not functional. Although the alternate pathway provides enough thymidylate for normal growth, the pool of thymidylate (or derivative) is, by our assumption, significantly lowered, "activating" the pathway for utilization of exogenous thymine. The functioning of this pathway is a necessary, but not sufficient, reason for the resistance of this strain to aminopterin in the presence of thymine. The resistance arises also from the sparing of THFA by loss of the thymidylate synthetase specified by the *thyA*$^+$ gene.

The mechanism of aminopterin selection of *thy* mutants in *B. subtilis* may be readily explained. A *thyA thyB*$^+$ mutant present in the population is, in contrast to the wild type, able to grow on the selective plate due to its conditional resistance to aminopterin, and ability to use exogenous thymine. The second mutation to *thyA thyB* confers further selective advantage, since strains with this genotype grow faster than *thyA thyB*$^+$ strains in aminopterin plus thymine medium.

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