Folate content of Bacillus subtilis

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FULATE CONTENT OF BACILLUS SUBTILIS

A Thesis
Presented to the
Department of Zoology
Brigham Young University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
David N. Hintze
April 1975
This thesis, by David N. Hintze, is accepted in its present form by the Department of Zoology of Brigham Young University as satisfying the thesis requirement for the degree of Master of Science.

Typed by Jacquelin Munns
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INTRODUCTION

Thymine is the only one of the five nucleic acid bases which is incorporated exclusively into deoxyribonucleic acid (DNA). The importance of understanding the biochemical synthesis of deoxythymidylate (dTMP) has been emphasized by workers using eukaryotic cells (Fridland, 1973), Escherichia coli (Werner, 1971), and Bacillus subtilis (Harris, 1973; Billen, Carreira, Hadden, and Silverstein, 1971). These studies showed that thymine deoxynucleotides are contained in two separate pools for the processes of DNA replication and repair synthesis, and they further suggested that such compounds may be involved in control of these vital cellular functions.

The de novo synthesis of dTMP requires deoxyuridine monophosphate (dUMP), a one-carbon donor, tetrahydrofolate (H$_4$-folate) and the action of thymidylate synthetase (Friedkin, 1973). Cells which require thymine are designated thy and are usually the result of a mutation at the thymidylate synthetase locus. However, Wilson, Farmer and Rothman (1966) found that the thy character in B. subtilis results from the necessary mutation of both of two unlinked genes designated thyA and thyB. The thyA gene product is thymidylate synthetase. The thyB gene product is thought to be an enzyme which catalyzes an alternate pathway for dTMP
synthesis, similar to thymidylate synthetase but likely requiring a different folate cofactor.

The existence of two pathways to manufacture the same product is not novel. Dual pathways for methylation reactions involving folate cofactors have been reported in *E. coli* (Woods, Foster and Guest, 1965). Other dual pathways have also been proposed for methyl transfer from $H_4$-folate to homocysteine in *Salmonella typhimurium* (Cauthen, Foster and Woods, 1966) and *Aerobacter aerogenes* (Morningstar and Kisliuk, 1965). It has also been reported that in *B. subtilis* there are two different enzymes which catalyze the oxidation of pyrroline to glutamine (Hauwer, Lavalle and Wiame, 1963).

Redundant pathways for the synthesis of cell metabolites are common enough that they might be expected to result from selection on gene duplications or mutations rather than from chance gene duplications or insertion of viral genes which have no selective advantage. Of the redundant pathways which have been reported, those responsible for dTMP synthesis in *B. subtilis* are particularly interesting because of the important role of dTTP in DNA synthesis and its possible role in regulation of cellular reproduction. There is experimental evidence for two separate pools of dTTP in bacterial cells, one used for DNA replication and the other for repair synthesis. If there is a critical functional reason for the existence of redundant pathways, it is important to investigate those pathways
under various conditions where they might be expected to be regulated.

Two groups of investigators reported that an alternative pathway for dTTP synthesis in two bacterial species might involve methylation and deamination of deoxycytidine triphosphate (dCTP) (O'Donovan and Neuhard, 1970). Later work has shown that the reaction sequence in question converts dCTP to dUMP, which is then a substrate of thymidylate synthetase. This pathway has been demonstrated in S. typhimurium (Neuhard and Thomassen, 1971), E. coli (O'Donovan, Edlin, Fuchs, Neuhard and Thomassen, 1971), and bacteriophage infected B. subtilis (Tomita and Takahashi, 1969). According to the last authors, the pathway does not exist in uninfected B. subtilis.

The possibility that there may be dual pathways for synthesis of dTMP has been reported for at least two organisms other than B. subtilis. Eisenstark, Eisenstark and Cunningham (1967) examined thy mutants of S. typhimurium and could not rule out the possibility that there might be two tightly linked thy loci. Chung, Ou, Rodriguez and Greenberg (1972) reported that E. coli may have a second pathway.

One of the necessary substrates for the thymidylate synthetase reaction is H$_4$-folate or one of its polyglutamate forms. Recently, an investigation by Kisliuk, Gaumont and Baugh (1974) demonstrated that tetrahydropteroyltriglutamate (H$_4$-PteGlu$_3$) and H$_4$-PteGlu$_6$ are both more effec-
tive substrates for thymidylate synthetase from *Lactobacillus casei* than is tetrahydrofolic acid (H$_4$PteGlu). Polyglutamate forms of folic acid are known to be the major forms of natural folates in animal tissues (Shin, Buehring and Stokstad, 1972), *L. casei* and *Streptococcus faecalis* (Buehring, Tamura and Stokstad, 1974), and numerous other organisms (Blakley, 1969). 5-formyltetrahydropteroyltrimet glutamate has been detected in *B. subtilis* by two different investigations (Hakala and Welch, 1957; Salem, Pattison and Foster, 1972). The latter group also reported the presence of a second, uncharacterized folate derivative in the same organism.

Since Wilson, Farmer and Rothman (1966) concluded that the *thyB* pathway for dTMP synthesis must have a different specificity for folates than the *thyA* pathway, obviously it would be of interest to isolate and characterize the folates from *B. subtilis* cells and to use these folates individually as the substrate for *in vitro* dTMP synthesis catalyzed by *thyA*<sup>+</sup>*thyB* and *thyAthyB*<sup>+</sup> soluble enzyme extracts, in order to determine which folate(s) is utilized in each of the postulated pathways.
MATERIALS AND METHODS

Growth Conditions

B. subtilis strains 23 thy, 168 thy ind (Farmer and Rothman, 1965), 168 met ileu leu thyA⁺thyB⁺, 168 met ileu leu thyA⁺thyB⁻ and 168 met ileu leu thyAthyB⁺ (Wilson, Farmer and Rothman, 1966) were grown in Difco Antibiotic Medium No. 3 at 37°C with aeration provided by shaking. Defined media were prepared from Spizizen’s minimal salts (Anagnostopulos and Spizizen, 1961) plus 0.5% glucose (SG). SG was sometimes supplemented with 1% monosodium glutamate (SGG). Supplements (thymine, L-isoleucine, L-leucine, and L-methionine) were normally added to a final concentration of 20 μg/ml when needed. Maximal growth rate was measured in a very rich synthetic medium composed of SG, 50 μg/ml each of the twenty common amino acids, uridine, inosine and a vitamin mixture, twenty times diluted from normal concentration (Hunt, 1970).

Extraction of Folates

B. subtilis strain 23 thy was grown in SGG plus thymine medium containing 2 μg/ml folic acid. When 1.5 liters of cells were in mid-exponential log growth (OD₅₄₀~1), they were harvested by centrifugation (4°C, 10,000 X g) for 30 minutes, resuspended in 20 ml SGG and
recentrifuged. The extraction procedure for folate compounds of Buehring, Tamura and Stokstad (1974) was followed. Cells were suspended in acetone which was 0.2 M in 2-mercaptoethanol and kept at 40°C for one hour under pure nitrogen. After centrifugation, the cell residues were washed once more with acetone-mercaptoethanol and again spun down. The cells were then resuspended in 0.01 M potassium phosphate buffer, pH 7.0 (0.2 M mercaptoethanol.) This was centrifuged (30 min., 4°C, 10,000 X g) and the supernatant was saved. The cells were washed in phosphate buffer once more and the cell residues were discarded. The combined phosphate buffer supernatant contained the folate compounds and it is subsequently referred to as "water extract."

**Treatment of Water Extract**

In order to have sufficient amounts of folate compounds for *in vitro* dTMP synthesis, it was necessary to concentrate the folates present in water extracts. To concentrate the folates, pure nitrogen gas was bubbled through 150 ml of water extract produced from 1.5 liters of cells. The extract was kept at 40°C during this evaporation. When the water extract had evaporated to a small volume (~5 ml) the residue was dissolved in 10 ml of 0.2 M mercaptoethanol. This is referred to as "concentrated water extract."

A few milliliters of the original, unconcentrated water extract were used for column fractionation to deter-
mine the elution patterns, and for cabbage conjugase treatment.

**Purification of Folate Compounds**

Portions of concentrated water extract were eluted from a Sephadex G-25 (fine) column (bed size 2.5 X 63 cm.) using 0.1 M potassium phosphate buffer, pH 7.0 which was 0.2 M in 2-mercaptoethanol as eluant. Each portion separated into two peaks of material active for the growth of *L. casei*. After the concentrated water extract had all been chromatographed on G-25 Sephadex, the fractions containing the two folate compounds were pooled and again concentrated by evaporation under nitrogen while heating to 45°. To desalt the concentrated material, it was applied to a QAE-Sephadex A-25 column (1.5 X 40 cm) previously equilibrated with 200 ml of 0.1 M triethylammonium bicarbonate, 0.2 M in mercaptoethanol (buffer). After application of the sample, the column was washed with 150 ml of 0.1 M buffer, and the folate compounds were then eluted with 200 ml of 1.0 M buffer. The buffer was then removed by evaporation under vacuum. The material which remained was dissolved in 2 ml of 0.01 M potassium phosphate buffer, pH 7.0 (0.2 M mercaptoethanol). The QAE Sephadex separation of folates has been described by Parker, Wu and Wood (1971).

**Microbiological Assay of Folate Compounds**

The microbiological assay procedure used was essentially that of Herbert (1966), which depends on the inabil-
ity of \textit{L. casei} to synthesize folic acid compounds.

An aliquot of the sample to be tested for folates was added to 5 ml of Folic Acid Assay PGA Broth (Baltimore Biological Laboratories). The Herbert procedure specifies that the folates to be tested should be added to the medium before autoclaving. To eliminate the possibility that autoclaving may in some way rearrange the folates and interfere with the accuracy of the assay, the same series of tubes was assayed in duplicate, adding the folates before autoclaving in one case, and adding the folates after autoclaving in the other case. Table 1 shows the results.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Folate Added Before Autoclaving} & \multicolumn{2}{c|}{\textbf{OD (640 nm)}} & \textbf{Folate Added After Autoclaving} & \multicolumn{1}{c|}{\textbf{OD (640 nm)}} \\
\hline
\textbf{Tube} & \textbf{Dilution} & \textbf{OD} & \textbf{Tube} & \textbf{Dilution} & \textbf{OD} \\
\hline
1 & undiluted & 1.495 & 1 & undiluted & 1.509 \\
2 & 10 X dil. & .821 & 2 & 10 X dil. & .870 \\
3 & 100 X dil. & .164 & 3 & 100 X dil. & .158 \\
4 & 1000 X dil. & .022 & 4 & 1000 X dil. & .022 \\
\hline
\end{tabular}
\caption{\textit{L. casei} Growth in Assay Medium}
\end{table}

As can be seen from Table 1, autoclaving the folates made no difference in their ability to support the growth of \textit{L. casei}, so to maintain the sterility of the assay medium,
folates were added to the assay medium before it was autoclaved and the *L. casei* inoculum was added after the medium had been cooled.

The inoculum was prepared as follows. Two drops of an *L. casei* culture in maintenance medium were added to 5 ml of assay medium. After incubation for six to eight hours, two drops of this fresh culture were added to a fresh tube of assay medium. After incubation at 37° for one or two hours, this tube was used as the inoculum and one drop of it was added to the assay tubes.

After incubation at 37° for 24 to 36 hours, the assay tubes were vortexed well, and the turbidity was determined spectrophotometrically at 640 nm. In all cases, the blank was an assay tube which had been inoculated with *L. casei* but had had no folates added.

**Chemicals**

Triethylammonium bicarbonate was prepared by neutralizing triethylamine with CO₂ in an ice bath until the pH was 7.5 (Parker, Wu and Wood, 1971).

Tetraopterin was a gift of Dr. Frank Rothman.

Tetrahydrofolic acid was a gift of Dr. John Mangum.

**Cabbage Conjugase**

Cabbage conjugase was prepared by the procedure of Tamura, Buehring and Stokstad (1972) with some modification. Twenty grams of a cabbage obtained at a local market were blended in 200 ml of 0.1 M potassium phosphate buffer,
pH 6.0 for a few seconds. Forty ml of the homogenate were centrifuged at 100,000 X g for one hour. The supernatant was then dialyzed, with several changes of the buffer, against a total of 780 ml of the same phosphate buffer used to remove folates.

Conjugase treatment of folates was done by combining 3.5 ml of sodium acetate buffer (0.1 M pH 4.8) containing 0.1% ascorbic acid, 1 ml of the folate containing material (water extract or column fraction) and 0.5 ml of the cabbage conjugase preparation. Tubes were incubated at 37° C for 20 to 38 hours.

Thymidylate Synthetase Assays

B. subtilis cells were grown in Difco Antibiotic Medium No. 3 until OD_{540} was approximately one. Crude cell extracts were prepared by grinding frozen cells in 0.05 M tris (hydroxymethyl) aminomethane plus 0.01 M 2-mercaptoethanol plus 0.001 M ethylenediaminetetraacetate (pH 7.4) with levigated alumina and were clarified by low-speed centrifugation followed by centrifugation at 100,000 X g for one hour. Assays were done as described by Wahba and Friedkin (1961).
RESULTS

Separation of Folates

Chromatographic fractionation of either water extract or concentrated water extract from \textit{B. subtilis} 23 \textit{thy} using a Sephadex G-25 column results in the separation of two peaks of material active for supporting the growth of \textit{L. casei}. Figure 1 shows a typical elution pattern of concentrated water extract and Figures 2 and 3 show the elution pattern of water extract.

The chromatographic behavior of folates has been described by Shin, Buehring and Stokstad (1972) who express the elution results as \( K_{av} \) values. \( K_{av} = \frac{V_e - V_o}{V_t - V_o} \) where \( V_o \) is the exclusion volume of the column determined as the elution volume of blue dextran 2000 (molecular weight 2,000,000), \( V_e \) the elution volume at which the concentration of the eluted substance is maximal, and \( V_t \) is the volume of the gel bed. The \( K_{av} \) values for the two folate derivatives of \textit{B. subtilis} are shown in Table 2. The published \( K_{av} \) values for PteGlu\(_3\), PteGlu\(_2\), and PteGlu were verified experimentally using a sample containing teropeterin (a commercial preparation of PteGlu\(_3\) which contains lesser amounts of several impurities, one of which is PteGlu\(_2\)), and folic acid. The elution pattern of these compounds from Sephadex G-25 is shown in Figure 4.
Figure 1. *L. casei* assay of the elution pattern of 2.0 ml concentrated water extract from a Sephadex G-25 column. Column dimension, 2.5 X 63 cm; flow rate 20 ml per hour; eluant 0.1 M potassium phosphate buffer, pH 7.0, with 0.2 M 2-mercaptoethanol. Fraction collecting began when blue dextran began to elute (125 ml from the time when the sample was placed on the column).
Figure 2. *L. casei* assay of the elution pattern from G-25 Sephadex of: ○-10 ml *B. subtilis* 23 thy water extract (equivalent to approximately $5 \times 10^9$ cells); □-2 ml of the same water extract treated with cabbage conjugase. Conjugase treatment combined 2 ml water extract with 7 ml sodium acetate buffer, pH 4.8 and 1 ml cabbage conjugase preparation. This mixture was incubated at $37^\circ$ for 24 hours. Column dimension, flow rate, and eluant are the same as Figure 1.
Figure 3. *L. casei* assay of the elution pattern from G-25 Sephadex of 10 ml of water extract from *B. subtilis* 23 thy. O—before the fractions were treated with cabbage conjugase. The conjugase treatment used 3.5 ml sodium acetate buffer (0.1 M containing 0.1% ascorbic acid, pH 4.8), 1.0 ml of the fraction, and 0.5 ml cabbage conjugase preparation. The conjugase treatment was done at 37°C for 19 hours. Column dimension, flow rate, and eluant are the same as Figure 1. △—after the same fractions were treated with cabbage conjugase.
Figure 4. *L. casei* assay of the elution pattern from G-25 Sephadex of folic acid and teropterin. Sample (volume 6.0 ml) contained 1 mg folic acid and 1 mg teropterin. Began collecting fractions when blue dextran began to elute. Column dimension, flow rate, and eluant are the same as Figure 1.
Table 2

$K_{av}$ Values of Folic Acids

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_{av}$</th>
<th>$K_{av}$ Values Reported*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em> folate 1</td>
<td>0.61</td>
<td>--</td>
</tr>
<tr>
<td><em>B. subtilis</em> folate 2</td>
<td>0.81</td>
<td>--</td>
</tr>
<tr>
<td>Teropterin</td>
<td>0.86</td>
<td>0.91 (PteGlu$_3$)</td>
</tr>
<tr>
<td></td>
<td>1.16</td>
<td>1.16 (PteGlu$_2$)</td>
</tr>
<tr>
<td>Folic acid</td>
<td>1.73</td>
<td>1.81 (PteGlu)</td>
</tr>
</tbody>
</table>

*Shin, Buehring and Stokstad, 1972.

Conjugase Treatment

Since *L. casei* grows very poorly on folates containing more than three glutamate residues (Tamura, Shin and Williams and Stokstad, 1972), an assay with *L. casei* of fractionated folates from any source is not likely to reveal the presence of the more highly conjugated forms of folates (PteGlu$_4$ or higher) unless the folates are treated with a conjugase ($\gamma$-glutamyl carboxypeptidase) preparation to cleave the folates down to a form which supports maximal growth of *L. casei*.

To be certain that in spite of dialyzation the cabbage conjugase preparation contained no folates from cabbage which would interfere with the assay of *B. subtilis* folates, dilutions were made of conjugase treated water extract and untreated water extract. These dilutions were
then assayed for their ability to support growth of \textit{L. casei}. Figure 5 shows these data. The conjugase treated dilutions of water extract were observed to have no additional folates. In fact, they supported \textit{L. casei} growth less well than the same amount of untreated water extract. This is probably due to some slight inhibition of \textit{L. casei} growth by the acetate buffer used in the conjugase treatment.

Water extract from \textit{B. subtilis} was treated with cabbage conjugase at pH 5. At this pH, cabbage conjugase splits pteroylpolyglutamates to monoglutamates (Tamura, Buehring and Stokstad, 1972). Figure 2 compares the elution patterns of untreated and cabbage conjugase treated water extract. As shown therein, the conjugase treated water extract shows a peak of material active for the growth of \textit{L. casei} in a position (tube 59) which would correspond to a 5-CHO-PteGlu (K\textsubscript{av}=1.44). Thus, the conjugase preparation is known to be active, since 5-CHO-PteGlu is known to be present in \textit{B. subtilis}. The peak of material active for supporting the growth of \textit{L. casei} which is evident in the region of tube 30 is presumed to be either polyglutamyl folate material which has not been cleaved by the cabbage conjugase or 10-CH\textsubscript{2}H\textsubscript{4}PteGlu which is reported to have a K\textsubscript{av} of 1.05.

Figure 3 shows the results obtained from cabbage conjugase treatment of individual fractions of water extract fractionated on G-25 Sephadex. As can be seen, no new peaks
Figure 5. *L. casei* assay of dilutions of water extract (O) and cabbage conjugase treated water extract (Δ). Diluted samples in 0.01 M potassium phosphate buffer, pH 7.0, with 0.2 M 2-mercaptoethanol. Used 10 μl of each dilution into 5 ml assay medium. One unit is arbitrarily defined as an amount equivalent to 10 μl of undiluted water extract.
are revealed, indicating that *B. subtilis* contains only two kinds of folates. PteGlu$_4$ or higher conjugated forms would be expected to elute closer to the void volume than PteGlu$_3$.

**Ability of One Pathway to Support Maximal Growth**

It was thought that one possible function of the second pathway might be to provide dTTP at increased rates during periods of rapid growth. To test this hypothesis, wild-type cells and cells lacking one or the other of the two pathways were grown in a very rich synthetic medium. The results are shown in Table 3.

**Table 3**

Growth Rates of *B. subtilis thyA$^+$thyB$^+$, thyA$^+$thyB, and thyAthyB$^+$

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Thymine Present in Medium</th>
<th>N</th>
<th>$\bar{x}$</th>
<th>$\sigma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>thyA$^+$thyB$^+$</td>
<td>No</td>
<td>4</td>
<td>27.8</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>27.6</td>
<td>2.9</td>
</tr>
<tr>
<td>thyA$^+$thyB</td>
<td>No</td>
<td>2</td>
<td>27.5</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>5</td>
<td>26.6</td>
<td>1.8</td>
</tr>
<tr>
<td>thyAthyB$^+$</td>
<td>No</td>
<td>1</td>
<td>26.0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td></td>
<td></td>
</tr>
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</table>

*Number of trials

**Mean number of minutes to double cell mass, as measured by light scattering.

***Standard deviation from the mean
Thymidylate Synthetase Assays

The assay for thymidylate synthetase activity depends upon the spectrophotometric changes in absorbancy of ultraviolet light by tetrahydrofolates as they are oxidized to dihydrofolates during the methylation of dUMP. After the two folates from *B. subtilis* were separated they failed to support any detectable thymidylate synthesis. The crude soluble enzyme preparations from *B. subtilis* 168 met ileu leu thyA thyB were active since they showed the expected activity when H₄-PteGlu was used as substrate.
DISCUSSION

Wilson, Farmer, and Rothman (1966) interpreted their data as being consistent with the hypothesis that the \textit{thyB}\textsuperscript{+} gene produce is an enzyme which catalyzes a reaction very similar to that catalyzed by thymidylate synthetase. The only major difference that was postulated was that the two enzymes have different specificities for the various polyglutamate forms of folate present in \textit{B. subtilis}. The hypothesis of Wilson, Farmer and Rothman predicts that thymidylate synthetase should use the reduced form of one or both of these folates as substrate while the \textit{thyB}\textsuperscript{+} gene product should utilize only one of them. (If thymidylate synthetase should utilize only one of the folates, the hypothesis predicts that the \textit{thyB}\textsuperscript{+} gene product will utilize a different folate.)

Previous investigations have shown \textit{B. subtilis} to contain at least two kinds of folate, one of which is 5-formyltetrahydropteroyltriglutamate (5-CHO\textit{H}\textsubscript{4}\textit{PteGlu}\textsubscript{3}) and the other is thought to be a diglutamate folate (Hakala and Welch, 1957; Salem, Pattison and Foster, 1972). It is clear from these papers that there is no pteroylmonoglutamate (PteGlu) in \textit{B. subtilis}, but the bioassays used by both investigations were not sensitive to folates with more than three glutamate residues, so the possibility that
*B. subtilis* might contain such highly conjugated folates was not excluded.

By using cabbage conjugase to treat *B. subtilis* folates, our investigation found no evidence that there are any folates present which cannot be detected by *L. casei* bioassay prior to such conjugase treatment. Thus, we were able to conclude that there are no folates with more than three glutamate residues present in *B. subtilis*.

Unambiguous identification of the two folate peaks revealed by Sephadex fractionation of *B. subtilis* water extracts cannot be made from our data. *B. subtilis* 23 thy was used as the source of folates in order to insure that the initial water extract contained reduced folates, (apparently the only reaction which oxidizes folates in substrate quantities is the synthesis of dTMP) but one of the folates is known to be N-substituted and the other may be. The description of chromatographic behavior of folates (Shin, Buehring and Stokstad, 1972) shows that unsubstituted folates capable of supporting the growth of *L. casei* (PteGlu$_3$ or smaller) elute from Sephadex G-25, under the conditions we employed, with $K_{av}$ values no less than 0.91. However, folate compounds substituted in the N5 or N10 position are less well absorbed on the gel and elute more rapidly than unsubstituted compounds. Since our data show that growth of *L. casei* is supported by material eluting at $K_{av}$ values with those estimated for substituted diglutamate and triglutamate folates, using the reported variations in
elution of N-substituted monoglutamate folates to make the estimate. Conjugase treatment of *B. subtilis* folates before column chromatography yielded material which eluted with a $K_{av}$ approximately equal to that reported for an N-substituted PteGlu, which indicated that prior to enzymatic cleavage, at least one of the polyglutamate folates of *B. subtilis* was N-substituted. The agreement of our data with that of Salem, Pattison, and Foster (1972) and Hakala and Welch (1957) in conjunction with the results of the experiment involving conjugase make it likely that there are only two folate compounds in *B. subtilis*, one a triglutamate and the other a diglutamate folate.

The ultimate goal of this research is, of course, to determine the substrate specificities of the folate cofactors in supporting dTMP synthesis by thymidylate synthetase and the thyB+ gene product. The reason for the failure of the isolated folates to support the *in vitro* synthesis of dTMP is not altogether clear. It is possible that in spite of every precaution the folates were oxidized and thus were not active enzymatically. It is also possible that the concentrations of folates were too low to assay dTMP synthesis spectrophotometrically, or that there has been some light-catalyzed rearrangement which made the folates inactive. The most likely explanation is that the folates which were N-substituted were not able to bind formaldehyde to form the true substrate of thymidylate synthetase.
The fact that cells lacking one or the other of the two pathways are phenotypically normal (with respect to mass increase at fast growth rates as measured by light scattering due to increased cell mass) indicates that either pathway can compensate for the loss of the other under those conditions which have been examined thus far.
LITERATURE CITED
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FOLATE CONTENT OF BACILLUS SUBTILIS

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ABSTRACT

Bacillus subtilis can synthesize deoxythymidylate via either of two pathways. The working hypothesis is that the two reactions are very similar, differing only with respect to specificity for the type of folate required as substrate.

The folates from B. subtilis were isolated and were determined by gel chromatography to be derivatives of pteroyltriglutamate and pteroyldiglutamate. Kav values for the two folate species are reported. Conjugase treatment revealed no other highly conjugated folates present.

It was also observed that mutants deficient in one or the other of the two pathways grow at the same rate as wild-type cells under conditions allowing very rapid growth in the absence of exogenous thymine.