JOURNAL OF BACTERIOLOGY, Jan. 1983, p. 316–325
0021-9193/83/010316-10$02.00/0
Copyright © 1983, American Society for Microbiology
Vol. 153, No. 1

Regulation of Folylpoly-γ-Glutamate Synthesis in Bacteria: In Vivo and In Vitro Synthesis of Pteroylpoly-γ-Glutamates by *Lactobacillus casei* and *Streptococcus faecalis*

BARRY SHANE,* ANDREW L. BOGNAR, REBECCA D. GOLDFARB, AND JONATHAN H. LEBOWITZ

Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland 21205

Received 13 August 1982/Accepted 21 October 1982

Lactobacillus casei and *Streptococcus faecalis* accumulated labeled folic acid and metabolized this compound to poly-γ-glutamates of chain lengths of up to 11 and 5, respectively. Octa- and nonaglutamates predominated in *L. casei*, and tetraglutamates predominated in *S. faecalis*. The most effective monoglutamate substrates for the *L. casei* and *S. faecalis* folylpoly-γ-glutamate (folylpolyglutamate) synthetases were methylene- and formyltetrahydrofolate, respectively. Methylenetetrahydropteroylpoly-γ-glutamates were the preferred poly-γ-glutamate substrates for both enzymes and, in each case, the highest activity was observed with the diglutamate substrate. The final distribution of folylpolyglutamates in these bacteria appeared to reflect the ability of folates with various glutamate chain lengths to act as substrates for the bacterial folylpolyglutamate synthetases. The proportions of individual folylpolyglutamates were markedly affected by culturing the bacteria in medium containing adenine, whereas thymine was without effect. Adenine did not affect the level of folylpolyglutamate synthetase in either organism but caused a large increase in the proportion of intracellular folates containing one-carbon units at the oxidation level of formate, folates which are substrates for enzymes involved in purine biosynthesis. The folates with shorter glutamate chain lengths in bacteria cultured in the presence of adenine resulted from primary regulation of the de novo purine biosynthetic pathway, regulation which caused an accumulation of formyltetrahydropteroylpoly-γ-glutamates (folate derivatives that are ineffective substrates for folylpolyglutamate synthetases), and did not result from regulation of folylpolyglutamate synthetase per se.

The major cellular forms of folate coenzymes are conjugated poly-γ-glutamate (polyglutamate) derivatives (10, 11, 16, 22). These coenzymes act as acceptors or donors of one-carbon units in a variety of reactions involved in amino acid and nucleotide metabolism (6). The physiological roles of folylpoly-γ-glutamates (folylpolyglutamates) in one-carbon metabolism have recently been reviewed (10, 11, 16, 22). Folylpolyglutamates are often better substrates than pteroylmonoglutamates for the enzymes of one-carbon metabolism and are preferentially retained by tissues, whereas pteroylmonoglutamates are the transport forms of the vitamin. In mammalian cells which lack the enzyme folylpolyglutamate synthetase, folate transport is unimpaired, but intracellular folate levels are reduced owing to an inability to synthesize folylpolyglutamates, and the mutant cells are auxotrophic for glycine, purines, thymidine, and methionine (21, 34).

Folates with different glutamate chain lengths are found in different organisms and tissues, and it has been suggested that the one-carbon flux through various folate-dependent reactions may be regulated under different growth conditions by varying the glutamate chain length of folates (1, 18). A major interest of our laboratory is to understand the mechanisms by which folylpolyglutamates of specific glutamate chain lengths are synthesized by different tissues and organisms and whether regulation of this process serves as a means for regulating one-carbon metabolism.

In this study, the in vivo and in vitro synthesis of folylpolyglutamates by *Lactobacillus casei* and *Streptococcus faecalis* were investigated, and the effects of additions of one-carbon-metabolism products to the culture medium on the intracellular distribution of folylpolyglutamates were assessed.

MATERIALS AND METHODS

Materials. [^{3}H]folic acid (pteroylmonoglutamate, PteGlu), labeled in positions 3', 5', 7, and 9 (29 Ci/mmol), (*dl*)-5-[^{14}C]methyltetrahydrofolate (5-[^{14}C]methyl-H_4PteGlu; 58 mCi/mmol), and L-[*U*-^{14}C]glutamate (10 mCi/mmol) were obtained from Amersham Corp.

$PteGlu_{2-7}$ (numbers indicate the total number of glutamate moieties), $PteGlu_3$-[^{14}C]Glu-Glu (0.5 mCi/mmol), and *p*-aminobenzoylpolyglutamates ($pABAglu_{2-7}$) were synthesized by the method of Baugh et al. (4). (*l*)-H_4[^{3}H]PteGlu (*l* indicates the natural diastereoisomer) and (*l*)-$H_4PteGlu_n$ were prepared by enzymatic reduction of [^{3}H]PteGlu and $PteGlu_n$ (17), using dihydrofolate reductase purified from methotrexate-resistant *L. casei*, as described previously by Whiteley et al. (35). (*l*)-10-Formyl-$H_4PteGlu_n$ was prepared from (*l*)-$H_4PteGlu_n$, using purified *Clostridium* 10-formyltetrahydrofolate synthetase (9). Other folate derivatives were prepared as described previously (9, 31). All folate derivatives were purified by DEAE-cellulose or QAE-Sephadex chromatography (31) and were stored in potassium phosphate buffer (pH 7) containing 200 mM β-mercaptoethanol at −196°C. The identity of each compound was confirmed, and its concentration assessed, by its absorption spectrum (5).

Cell culture and labeling of intracellular folates. *L. casei* (ATCC 7469) and *S. faecalis* (ATCC 8043) were cultured at 37°C in a complex defined medium containing 2.3 nM PteGlu, as described previously (30). The medium contained serine (ca. 3 mM), glycine (ca. 1.3 mM), glutamate (ca. 9 mM), and methionine (ca. 1.1 mM), all derived from a pancreatin digest of casein, and uracil (45 μM), but lacked purines and thymine. Under these conditions, folate was required primarily for the biosynthesis of purines and thymidylate (32, 33).

Bacteria were harvested from growth medium and washed twice with 0.9% (wt/vol) NaCl, and a sample was used to inoculate medium (10 ml) containing 17 nM [^{3}H]PteGlu (0.5 μCi/ml). Where indicated, adenine (1 mM), thymine (50 μM), or both were added at levels that were sufficient to overcome the folate requirements of the organisms for the biosynthesis of these compounds (33). The cultures were incubated at 37°C for 20 to 22 h, and late-log phase bacteria were collected by centrifugation. The bacteria were washed three times with ice-cold 0.9% (wt/vol) NaCl and suspended in 20 mM sodium phosphate buffer (pH 6.5) containing 50 mM β-mercaptoethanol. A sample of the suspension, to be used for the determination of polyglutamate chain length, was heated at 100°C for 6 min to release intracellular folates, and, after the sample cooled to 4°C, debris was removed by centrifugation. The remainder of the suspension, which was utilized for the determination of folate one-carbon forms, was treated in a similar fashion, except that sodium ascorbate buffer (pH 6; final concentration, 10 mM) was added before the heating step. Extracts were stored under N_2 at −196°C until used.

Identification of polyglutamate chain length. Folates in cell extracts were cleaved to $pABAglu_n$ and a pterin derivative by a procedure that allows the quantitative conversion of all naturally occurring folates to $pABAglu_n$ (13). The resulting $pABAglu_n$'s were converted to azo dyes of naphthylethylenediamine which were purified and separated from the pterin derivative by chromatography on Bio-Gel P2 (7). The purified azo dyes were reductively reconverted to $pABAglu_n$ by treatment with Zn under acidic conditions, unlabeled $pABAglu_n$ standards were added, and the solutions were adjusted to pH 6.5 and clarified by filtration (29). Individual polyglutamates were separated by high-performance liquid chromatography (HPLC) on a column (250 by 4.6 mm inner diameter) containing a strong anionic exchanger (Whatman SAX), as described previously (29). Labeled derivatives were detected by scintillation counting with a Beckman LS7000 scintillation counter (tritium counting efficiency, ca. 30%), and unlabeled standards were detected by monitoring the absorbance at 280 nm (A_{280}).

Identification of one-carbon forms. Labeled intracellular folates were converted to monoglutamate derivatives by treatment with hog kidney γ-glutamyl hydrolase (conjugase) (27). The reaction mixtures contained the following: bacterial extract (250 μl, containing 0.8 to 13 pmol of labeled folate); sodium acetate buffer, pH 4.5 (33 mM); glutamate (67 μM); sodium ascorbate buffer, pH 4.2 (20 mM); and γ-glutamyl hydrolase (35 U) in a total volume of 1.5 ml. The mixtures were incubated at 37°C for 4 h. One unit of enzyme released 1 nmol of [^{14}C]glutamate per h when 17 μM Pte-Glu_3-[^{14}C]Glu-Glu was used as the folate substrate in the assay mixture described above.

The samples were adjusted to pH 6.5 with NaOH and centrifuged, and the supernatants were diluted 10-fold with 0.2 M mercaptoethanol. A variety of folate standards were added, and the samples were applied to DEAE-cellulose columns (20 by 1 cm; Serva DE 23SS) that had been preequilibrated with 10 mM sodium phosphate buffer (pH 6) containing 0.2 M mercaptoethanol. The columns were eluted with an exponential phosphate gradient formed with 100 ml of 10 mM sodium phosphate (pH 6)–0.2 M mercaptoethanol in a closed mixing chamber attached to a reservoir of 500 mM sodium phosphate (pH 6)–0.2 M mercaptoethanol. The standards used were 10-formyl-H_4PteGlu and 5-formyl-H_4PteGlu (detected by the A_{350} after acidification), pABAglu (detected by the A_{550} of its azo dye derivative), 10-formyl-PteGlu and PteGlu (detected by the A_{347}), H_4PteGlu (detected by the A_{297}), and 5-[^{14}C]methyl-H_4PteGlu (detected by scintillation counting).

Folylpolyglutamate synthetase extraction and assay. Bacteria were collected from growth medium by centrifugation, washed three times with ice-cold 0.9% (wt/vol) NaCl, and suspended in 50 mM potassium phosphate buffer (pH 7) containing 50 mM KCl at 4°C. Enzyme was extracted by sonication for 10 min with a Branson W-350 sonicator at a setting of 8 in the pulsed mode (20 min at 0.5 s/s). The sonicate was centrifuged, and the supernatant was dialyzed overnight against 50 mM potassium phosphate buffer (pH 7) containing 50 mM KCl (100 volumes) to give a crude enzyme extract.

The crude extract was concentrated and partially purified by ammonium sulfate fractionation. Protein precipitating between 20- and 50%-saturated ammonium sulfate was resuspended in the dialysis buffer and dialyzed overnight against the same buffer.

Folylpolyglutamate synthetase was routinely assayed by the incorporation of [^{14}C]glutamate into

folylpolyglutamates as described previously for the *Corynebacterium* enzyme (28). The reaction mixture contained the following: 100 mM tris–50 mM glycine buffer (pH 10), KCl (200 mM), dithiothreitol (5 mM), mercaptoethanol (10 mM; derived from folate solution), bovine serum albumin (50 μg), dimethyl sulfoxide (50 μl), $MgCl_2$ (10 mM), ATP (5 mM), L-[^{14}C]glutamate (250 μM; 1.25 μCi), folate (100 μM), and enzyme preparation in a total volume of 0.5 ml. (*dl*)-5,10-Methylene-H_4PteGlu, formed by adding (*dl*)-H_4PteGlu and formaldehyde (5 mM) to the assay mixture, was normally used as the folate substrate for the *L. casei* enzyme, and (*dl*)-10-formyl-H_4PteGlu was used for the *S. faecalis* enzyme. Reaction tubes were capped and incubated at 37°C for 2 h. The reaction was stopped by the addition of ice-cold 30 mM mercaptoethanol (1.5 ml).

Folate product was separated from unreacted labeled glutamate by a modification (28) of the procedure of McGuire et al. (23). The reaction mixture was applied to a DEAE-cellulose (DE52, Whatman Inc.) column (2 by 0.7 cm) protected by a 3-mm layer of nonionic cellulose and allowed to drain in. The column was washed three times, each time with 5 ml of 10 mM tris buffer (pH 7.5) containing 80 mM NaCl, to remove labeled glutamate, and the labeled product was eluted with 0.1 N HCl (3 ml).

For studies on the effects of folate concentration on the in vitro synthesis of folylpolyglutamates, the *L. casei* assay mixture was modified by the use of (*l*)-H_4[^{3}H]PteGlu as the folate substrate, and unlabeled glutamate (5 mM) replaced [^{14}C]glutamate. In these cases, the 0.1 N HCl eluates from the DE52 columns were treated with $HgCl_2$ to remove mercaptoethanol, and the solutions were acidified to effect cleavage of the products to $pABAglu_n$. The labeled $pABAglu_n$ derivatives were identified and quantitated by HPLC analysis after their purification as azo dye derivatives as described above.

Protein assays. Protein concentration was measured by the method of Lowry et al. (19), using bovine serum albumin as a standard.

RESULTS

Identification of labeled folate metabolites. Figures 1 and 2 show typical HPLC elution profiles of labeled $pABAglu_n$ derived from labeled folates in *L. casei* and *S. faecalis*. The folate cleavage procedure used in this study has been described elsewhere (13, 29). Over 95% of the label in the 3′ and 5′ positions of the folate molecules was recovered as $pABAglu_n$ derivatives, using the described procedures for the cleavage and separation of bacterial folate metabolites (13, 29).

The major folates in *L. casei* were octa- and nonaglutamates, and polyglutamates of chain lengths of up to 11 were detected (Fig. 1). Although $pABAglu_{8\text{-}11}$ standards were not available, labeled peaks eluting after the position of $pABAglu_7$ were assigned as $pABAglu_{8\text{-}11}$, based on their prior purification as azo dye derivatives, which indicated that they were primary aromatic amines. In addition, γ-glutamyl hydrolase treatment of the *L. casei* extracts before the folate cleavage procedure resulted in the loss of the $pABAglu_{2\text{-}7}$ and presumptive $pABAglu_{8\text{-}11}$ peaks and a concomitant increase in the pABAglu peak.

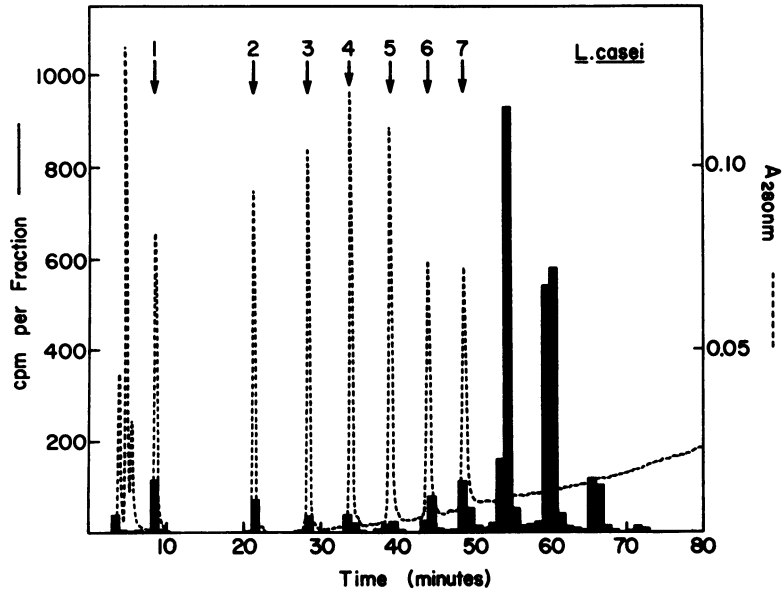

FIG. 1. HPLC elution profile of labeled $pABAglu_n$ derived from *L. casei* folates after the bacteria were cultured for 22 h in medium lacking adenine and thymine. Experimental conditions are given in the text. The numbers 1 to 7 indicate the elution positions of $pABAglu_{1\text{-}7}$ standards, which were detected by the A_{280}.

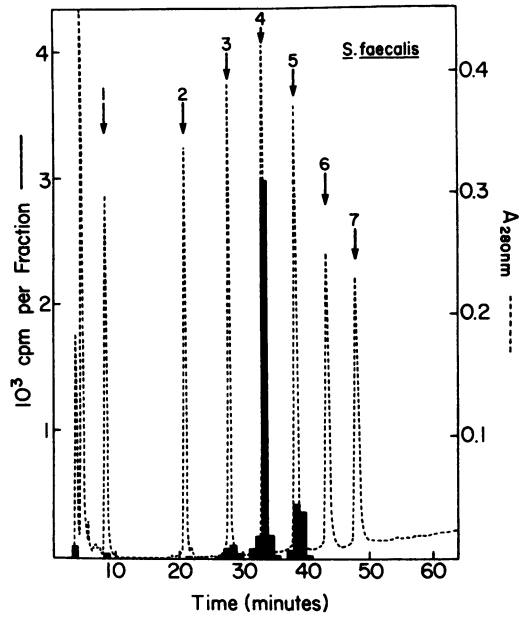

FIG. 2. HPLC elution profile of labeled $pABAglu_n$ derived from *S. faecalis* folates after the bacteria were cultured in medium lacking adenine and thymine. Experimental conditions are described in the legend to Fig. 1.

S. faecalis contained labeled folates of glutamate chain lengths of up to five, with the tetraglutamate predominating (Fig. 2).

Effect of one-carbon-metabolism products on distribution of folylpolyglutamates. *L. casei* and *S. faecalis* cultured under the described conditions required folate primarily for the synthesis of purines and thymidylate (32, 33). Addition of adenine or thymine (or both) to the *L. casei* culture medium did not affect the accumulation of [^{3}H]PteGlu or the growth rate of the organism (Table 1). Adenine, however, had a marked effect on the accumulation of [^{3}H]PteGlu by *S. faecalis* (Table 1). The decreased accumulation of labeled folate by *S. faecalis* when cultured in the presence of adenine correlated with a decreased cell yield and did not appear to be due to an effect of adenine on folate transport per se.

Thymine had no significant effect on the distribution of labeled folylpolyglutamates in *L. casei* or *S. faecalis* (Table 2). Adenine, however, caused a small, but significant, decrease in the average glutamate chain length of folates in both organisms. The decrease was very marked for the folates with longer glutamate chain lengths. For instance, the proportions of undeca-, deca-, and nonaglutamate in *L. casei* decreased by 100, 81, and 35%, respectively, whereas octa-, hepta-, and hexaglutamates increased by 21, 208, and 47%, respectively. Similarly, in *S. faecalis*, adenine caused 27 and 18% decreases in the proportions of penta- and tetraglutamates, whereas the proportion of triglutamate increased 317%.

These experiments have been repeated three times. Small differences have been observed among the average glutamate chain lengths of folylpolyglutamates from experiment to experiment. However, in each case, adenine caused a dramatic decrease in the proportions of folates

TABLE 1. Net accumulation of [^{3}H]PteGlu by *L. casei* and *S. faecalis*[a]

Bacterium	Net uptake (%) of [^{3}H]PteGlu with the following addition[b]:			
	None	Adenine	Thymine	Adenine + thymine
L. casei	53.2 ± 8.0	52.8 ± 9.0	57.3 ± 6.4	53.5 ± 7.8
S. faecalis	10.3 ± 1.4	3.3 ± 0.7	10.0 ± 0.5	3.7 ± 0.7

[a] Bacteria were cultured for 22 h at 37°C in medium containing the indicated additions and [^{3}H]PteGlu (17 nM), as described in the text.

[b] Values are mean ± standard deviation for three experiments.

TABLE 2. Effect of adenine and thymine on the distribution of folylpolyglutamates in bacteria[a]

Glutamate chain length	% Polyglutamate distribution with the following additions:			
	None	Adenine	Thymine	Adenine + thymine
L. casei				
1	4.4	3.8	3.7	3.5
2	2.6	2.0	2.2	1.0
3	0.5	0.6	1.0	0.3
4	1.4	1.1	1.7	1.5
5	1.0	0.9	1.4	1.0
6	3.6	5.2	3.2	4.8
7	5.6	18.3	5.5	15.9
8	37.6	43.6	36.1	45.9
9	36.5	23.0	37.3	25.0
10	6.3	1.5	7.1	1.1
11	0.5	0	0.6	0
Mean	7.80	7.49	7.85	7.61
S. faecalis				
1	0.9	1.5	0.7	3.2
2	0.5	0.6	0.4	2.0
3	6.0	20.2	4.2	22.3
4	77.3	67.9	75.7	57.4
5	15.3	9.8	19.0	15.1
Mean	4.06	3.84	4.12	3.79

[a] Bacteria were cultured for 22 h at 37°C in medium containing [^{3}H]PteGlu (17 nM) and the indicated additions. Intracellular folates were extracted and converted to $pABAglu_n$ and separated by HPLC as described in the text and shown in Fig. 1 and 2.

with longer glutamate chain lengths, whereas thymine was without effect.

Effect of one-carbon-metabolism products on folate one-carbon pool. Labeled folate one-carbon derivatives in *L. casei* and *S. faecalis* were identified by chromatographing γ-glutamyl hydrolase-treated intracellular folate extracts on DEAE-cellulose (Fig. 3). Bacterial extracts contained an unidentified γ-glutamyl hydrolase inhibitor, and large amounts of enzyme were required for the complete hydrolysis of folates to monoglutamate derivatives. In both organisms, the major intracellular folates were 5-formyl-H_4PteGlu, 10-formyl-H_4PteGlu, and H_4PteGlu derivatives. The predominant one-carbon derivative in *L. casei* cultured in basal medium was H_4PteGlu, whereas formyl derivatives predominated in *S. faecalis* (Fig. 3; Table 3).

Under the described extraction and separation conditions used, 5,10-methylene-H_4PteGlu was converted to H_4PteGlu and 10-formyl-H_4PteGlu was partially converted to 5-formyl-H_4PteGlu. It is not possible to say whether these organisms contained 5-formyl-H_4PteGlu or whether this one-carbon derivative arose by interconversion of 10-formyl-H_4PteGlu under the described experimental conditions.

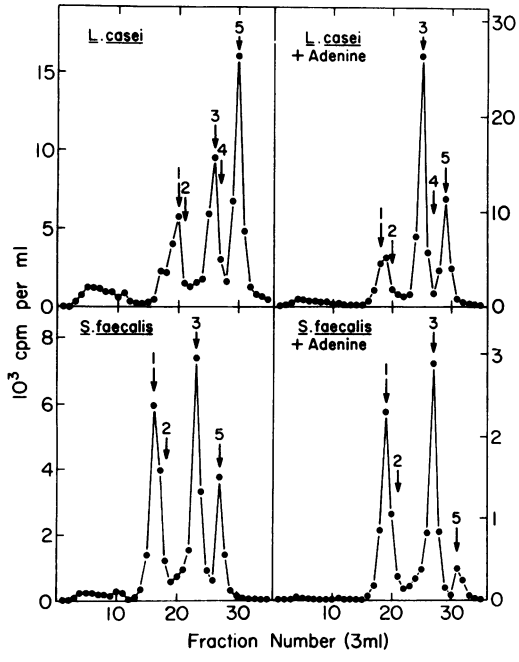

FIG. 3. DEAE-cellulose elution profiles of labeled bacterial folates after treatment with γ-glutamyl hydrolase. Experimental conditions are given in the text. The elution positions of standards chromatographed with the bacterial extracts are indicated as follows: (1) 10-Formyl-H_4PteGlu, (2) pABAglu, (3) 5-formyl-H_4PteGlu, (4) 5-methyl-H_4PteGlu, and (5) H_4PteGlu.

TABLE 3. Effect of adenine and thymine on the distribution of folate one-carbon forms in bacteria[a]

Folate	% Polyglutamate distribution with the following additions:			
	None	Adenine	Thymine	Adenine + thymine
L. casei				
10-Formyl-H_4PteGlu	12.4	9.0	14.4	10.4
5-Formyl-H_4PteGlu	29.6	52.7	30.2	58.9
H_4PteGlu	43.8	26.4	43.2	20.9
10-Formyl-PteGlu	3.9	3.0	3.8	2.6
pABAglu	10.2	8.9	8.4	7.2
S. faecalis				
10-Formyl-H_4PteGlu	33.8	39.8	34.2	33.3
5-Formyl-H_4PteGlu	40.7	48.1	40.2	47.6
H_4PteGlu	18.1	6.7	16.8	11.0
10-Formyl-PteGlu	2.1	2.9	4.2	3.8
pABAglu	5.3	2.6	4.6	4.5

[a] Experimental conditions are described in the legend to Table 2. Labeled intracellular folates were converted to monoglutamate derivatives by treatment with γ-glutamyl hydrolase and were chromatographed on DEAE-cellulose as described in the text and shown in Fig. 3.

L. casei has been reported to contain significant amounts of 5-methyl-H_4PteGlu, although this one-carbon derivative is not found in *S. faecalis* (8). Although the chromatographic procedure used in this study did not completely resolve 5-formyl-H_4PteGlu from 5-methyl-H_4PteGlu (Fig. 3), it was clear that the major derivative eluting in this region eluted at the position of 5-formyl-H_4PteGlu and that *L. casei* contained little, if any, 5-methyl-H_4PteGlu$_n$.

Addition of thymine to the culture media of both bacteria did not significantly affect the distribution of one-carbon forms of folate. However, adenine caused a dramatic redistribution of one-carbon derivatives (Fig. 3). The proportion of H_4PteGlu was reduced an average of 46% in *L. casei* and 49% in *S. faecalis*, with a concomitant increase in the proportion of formyl derivatives in both organisms (Table 3).

Substrates of bacterial folylpolyglutamate synthetases. Mono- and polyglutamyl folate substrate specificities of the *L. casei* and *S. faecalis* folylpolyglutamate synthetases were assessed with enzyme extracts partially purified as described above (Table 4). Both bacteria lacked γ-glutamyl hydrolase activity.

5,10-Methylene-H_4PteGlu was the preferred monoglutamate substrate for the *L. casei* enzyme, and other pteroylmonoglutamates were utilized less effectively. 5,10-Methylene-H_4PteGlu$_n$'s were also the preferred polyglutamate substrates for the enzyme. The large drop

TABLE 4. Folate substrates of bacterial folylpolyglutamate synthetases[a]

Glutamate chain length	Relative activity of folylpolyglutamate synthetase with the following substrate:		
	H_4PteGlu	5,10-Methylene-H_4PteGlu	10-Formyl-H_4PteGlu
L. casei			
1	30	100[b]	9
2	48	303	27
3	17	81	17
4	8	10	1
5	2	6	0
6	0	7	0
7	0	3	0
S. faecalis			
1	21	20	100[c]
2	14	197	27
3	1	20	0
4	0	1	0
5	0	0	0

[a] Folylpolyglutamate synthetase activity was measured as described in the text, using the indicated (*l*)-isomers (50 μM) as substrates and bacterial enzyme partially purified by ammonium sulfate fractionation. Activities are expressed relative to that obtained with the preferred PteGlu substrate.

[b] 532 pmol/h per mg of protein.

[c] 741 pmol/h per mg of protein.

in substrate effectiveness between tri- and tetraglutamate substrates and the gradual drop with further glutamate chain elongation suggests that tetra- or pentaglutamate derivatives should build up in this organism, which is not in keeping with the observed in vivo distribution of folylpolyglutamates (Fig. 1; Table 2). This point will be addressed below.

10-Formyl-H_4PteGlu was the preferred monoglutamate substrate for the *S. faecalis* enzyme (Table 4). However, 10-formyl-H_4PteGlu$_n$ polyglutamate derivatives were poor substrates or lacked activity. The preferred, and sometimes only, polyglutamate substrates were 5,10-methylene-H_4PteGlu$_n$ derivatives. The large drop in substrate effectiveness between 5,10-methylene-H_4PteGlu$_3$ and 5,10-methylene-H_4PteGlu$_4$ is consistent with the in vivo buildup of tetraglutamate derivatives in this organism (Fig. 2; Table 2).

Effects of one-carbon-metabolism products on folylpolyglutamate synthetase activity. Folylpolyglutamate synthetase activity in crude extracts of *L. casei* and *S. faecalis* was relatively unaffected by culturing the cells in media containing adenine or thymine (Table 5). Enzyme levels were also unaffected by variations of the PteGlu concentration of the medium or when bacteria were cultured in media lacking folate.

In vitro synthesis of folylpolyglutamates by *L. casei*. *L. casei* folylpolyglutamate synthetase was assayed with labeled folate monoglutamate substrates and unlabeled glutamate, and the labeled products were identified by HPLC analysis (Table 6). Extending the incubation time from 3 to 24 h did not significantly affect the amount of product formed, suggesting that little active enzyme remained after 3 h.

With (*l*)-5,10-methylene-H_4PteGlu as the substrate, at concentrations ranging from 1 nM to 20 μM, products with glutamate chain lengths of up to tetraglutamate were detected. In most cases, the major product was triglutamate, which is consistent with the high substrate effectiveness of 5,10-methylene-H_4PteGlu$_2$ (Table 4).

(*l*)-H_4PteGlu was a less effective substrate and was converted to di- and triglutamate products, but no tetraglutamate was detected (Table 6).

DISCUSSION

Although folylpolyglutamates were originally thought to be intracellular storage forms of folate, their physiological role as cofactors for the enzymes involved in one-carbon metabolism is now fairly well established (10, 11, 16, 22). However, little information is available on the in vitro synthesis of folylpolyglutamates in bacteria or the possible role of regulation of this synthesis as a mechanism by which one-carbon metabolism may be regulated. In this report, recently developed techniques for the identification of folylpolyglutamates have been used to study the

TABLE 5. Effect of growth medium on folylpolyglutamate synthetase levels[a]

Addition(s) (+)/deletion(s) (−)	Relative sp act	
	L. casei	*S. faecalis*
None	100[b]	100[c]
+ Adenine	105	81
+ Thymine	99	120
+ Adenine, + thymine	96	97
− PteGlu, + adenine, + thymine	90	137
+ 0.23 nM PteGlu	110	110
+ 22.7 nM PteGlu	103	115
+ 227 nM PteGlu	78	139
+ 2.27 μM PteGlu	115	122

[a] Bacteria were cultured for 22 h in basal medium with the indicated additions or deletions. Crude enzyme extracts were prepared by sonication and centrifugation. Folylpolyglutamate synthetase activity was measured as described in the text, using 100 μM 5,10-methylene-H_4PteGlu (*L. casei*) or 100 μM 10-formyl-H_4PteGlu (*S. faecalis*) as the folate substrate. The basal medium contained 2.27 nM PteGlu. PteGlu concentrations, where indicated, are final concentrations.

[b] 451 pmol/h per mg of protein.

[c] 620 pmol/h per mg of protein.

TABLE 6. In vitro synthesis of folylpolyglutamates by *L. casei*[a]

Folate substrate	Incubation time (h)	Product formed (pmol) with glutamate chain lengths of:		
		2	3	4
5,10-Methylene-H_4PteGlu				
2 μM	3	75	155	23
2 μM	24	65	202	43
10 μM	3	378	500	74
10 μM	24	373	532	96
20 μM	3	801	907	84
20 μM	24	684	953	94
1 nM	3	0.10	0.17	0.06
10 nM	3	1.2	1.6	0.4
100 nM	3	10.0	14.6	2.7
1 μM	3	70.8	49.4	3.5
H_4PteGlu				
1 nM	3	0	0	0
10 nM	3	0.04	0.02	0
100 nM	3	0.44	0.24	0
1 μM	3	2.5	0.4	0

[a] Partially purified *L. casei* folylpolyglutamate synthetase (1.2 mg of protein) was assayed as described in the text, except that the assay mixture (0.5 ml) contained (*l*)-H_4[^{3}H]PteGlu or (*l*)-5,10-methylene-H_4[^{3}H]PteGlu, as indicated, as the folate substrate, and unlabeled glutamate replaced [^{14}C]glutamate. The mixtures were incubated at 37°C for the indicated times. The labeled folate products were converted to pABAglu$_n$, which was quantitated after HPLC separation as described in the text.

in vivo and in vitro synthesis of folylpolyglutamates in bacteria, and the potential regulation of this process by products of one-carbon metabolism.

The most effective PteGlu substrates for the *L. casei* and *S. faecalis* folylpolyglutamate synthetases were 5,10-methylene-H_4PteGlu and 10-formyl-H_4PteGlu, respectively. However, for both bacteria, the preferred, and sometimes only, polyglutamate substrates were 5,10-methylene-H_4PteGlu$_n$'s. In each case, more activity was observed with the diglutamate substrate than with the monoglutamate. This substrate specificity is similar to that observed with the *Corynebacterium* enzyme, which preferentially utilizes H_4PteGlu as its monoglutamate substrate and 5,10-methylene-H_4PteGlu$_n$'s as its polyglutamate substrates (10, 28) and differs from mammalian folylpolyglutamate synthetases which preferentially use H_4PteGlu$_n$'s as their polyglutamate substrates (10, 22).

The in vivo distribution of folylpolyglutamates in *S. faecalis* reflected the ability of folate derivatives to act as substrates for the folylpolyglutamate synthetase of the organism. 5,10-Methylene-H_4PteGlu$_3$ was an effective substrate, whereas tetraglutamate derivatives were very poor substrates. Polyglutamates of chain lengths of four or longer were also fairly poor substrates for the *L. casei* synthetase, suggesting that tetra- or pentaglutamates should predominate in vivo. However, in this organism, octa- and nonaglutamates were the major intracellular folates. In addition, crude extracts of *L. casei* metabolized labeled 5,10-methylene-H_4PteGlu up to the tetraglutamate, but derivatives with longer polyglutamate chain lengths were not detected, even at very low (1 nM) substrate levels. It should be noted that, under the experimental conditions used, most of the PteGlu substrate was not converted to product, as enzyme activity was low in crude *L. casei* extracts and was unstable with prolonged incubation.

These data suggest either that a separate enzyme is responsible for the elongation of the glutamate chains of folates with longer chain lengths in *L. casei* or that the affinities of the derivatives with longer glutamate chain lengths for a single enzyme are much poorer than the affinities of the mono-, di-, and triglutamate substrates for the enzyme. All attempts at detecting a second folylpolyglutamate synthetase activity have been unsuccessful. In addition, we have recently purified the *L. casei* folylpolyglutamate synthetase to homogeneity (A. Bognar and B. Shane, manuscript in preparation). The substrate specificity of the purified enzyme is similar to that reported here for partially purified enzyme, with 5,10-methylene-H_4PteGlu$_{5,6,7}$ demonstrating low, but detectable, activities as substrates. These data are best explained by a single enzyme activity with considerably reduced affinity for folates with longer glutamate chain lengths. Previous studies have shown that the folylpolyglutamate distribution in *L. casei* is dependent upon the concentration of folate in the culture medium and that pteroyltetra- and pteroylpentaglutamates predominate in vivo when the bacteria are cultured in medium containing high folate levels (800 nM; 2, 26). In the present study, the initial folate concentration in the medium was 17 nM, which is about 10-fold higher than that required for maximal growth rates of *L. casei*, and the intracellular total vitamin concentration after 22 h of culture was about 2.3 μM. Total intracellular folate after 22 h corresponded to approximately 18 pmol/mg of bacterial protein. The specific activities of folylpolyglutamate synthetase in crude extracts of *L. casei* with 5,10-methylene-H_4PteGlu$_{1,2,3}$ as substrates (ca. 450, 1,360, and 360 pmol of product/h per mg of protein) were considerably in excess of those required to account for the in

vivo metabolism of the labeled folate tracer. The specific activities with penta-, hexa-, and heptaglutamate substrates (10 to 30 pmol/h per mg) were also more than sufficient to account for the in vivo metabolism of the tracer to octa- and nonoglutamate derivatives. It appears that under normal physiological conditions, PteGlu's can be metabolized to long-chain polyglutamate derivatives. However, under conditions which tax the ability of the cells to metabolize folate derivatives with shorter glutamate chain lengths, e.g., high medium folate levels, the accumulation of these compounds inhibits chain elongation of the derivatives with longer polyglutamate chain lengths.

Studies on the regulation of one-carbon metabolism have usually involved studying the effects of different folate derivatives and products of one-carbon metabolism on the levels and activities of individual folate-requiring enzymes (11, 16, 20, 22, 24, 25). In recent years, folylpolyglutamates have been demonstrated to be more effective than monoglutamates as substrates for many of the enzymes involved in one-carbon metabolism (10, 11, 16, 22). In addition, some folylpolyglutamates are very effective inhibitors of a number of folate-requiring enzymes (10, 11, 16, 22), suggesting a regulatory role for these compounds in one-carbon metabolism. This has led to the hypothesis that one-carbon metabolism may be regulated by changing the glutamate chain length of intracellular folates under different conditions of growth or nutritional requirement (1, 18).

If one-carbon metabolism is regulated by changes in the glutamate chain length of folates, changes in the distribution of folylpolyglutamates should be detectable under conditions which change the requirements for products of one-carbon metabolism. Such differences have been observed in *Escherichia coli* between stationary phase and exponentially growing cells (14), and when products of one-carbon metabolism (i.e., thymidine, adenosine, methionine, and serine) are added to the culture medium (3). Similarly, we found that adenine, but not thymine, caused a dramatic decrease in the proportion of folates with longer chain lengths in *L. casei* and *S. faecalis*, and significantly impaired the growth of *S. faecalis*.

The changes in the distribution of folylpolyglutamates under some nutritional conditions which have been observed in this study as well as in others are consistent with the hypothesis that one-carbon metabolism is regulated by changes in the glutamate chain length of folates. However, the observed changes might also be explained by changes in the metabolic flux through various folate-requiring reactions under different nutritional conditions, causing changes in the one-carbon folate distribution in the cell. In such a case, differences in the rate of synthesis of folylpolyglutamates would be expected, owing to differences in the ability of different one-carbon forms of folate to act as substrates for folylpolyglutamate synthetase. This appears to be the case in the present study, as the adenine-induced shift in the distribution of folylpolyglutamates in *L. casei* and *S. faecalis* can be entirely explained by its effect on the folate one-carbon pool. In both bacteria, adenine caused a dramatic increase in the proportion of formyl folate derivatives, which are poor substrates for folylpolyglutamate synthetase. In addition, if direct manipulation of the polyglutamate chain length of folates is a means of regulating one-carbon metabolism, some mechanism would have to exist to modulate the glutamate chain length, either a change in the activity of folylpolyglutamate synthetase or, possibly, a change in the activity of γ-glutamyl hydrolase. However, these bacteria lack γ-glutamyl hydrolase activity, and the specific activity of folylpolyglutamate synthetase in crude bacterial extracts was unaffected by addition of adenine or thymine to the culture medium or by large variations in the medium folate concentration.

Folate, in the form of 10-formyl-$H_4PteGlu_n$, is involved in two steps of the de novo purine biosynthetic pathway. The buildup of these derivatives in bacteria cultured in the presence of adenine reflects their decreased utilization due to primary regulation of the purine biosynthetic pathway. Serine and glycine are the major sources of one-carbon units in bacteria, which enter the folate pool as 5,10-methylene-$H_4PteGlu_n$ (12). 5,10-Methylene-$H_4PteGlu_n$ can be converted to 10-formyl-$H_4PteGlu_n$ in reactions catalyzed by methylenetetrahydrofolate dehydrogenase and cyclohydrolase. The dehydrogenase is partially repressed in bacteria cultured in the presence of purines and is noncompetitively inhibited by 10-formyl-$H_4PteGlu$ (15). Computer simulations of one-carbon metabolism in *E. coli* have suggested that any accumulation of 10-formyl-$H_4PteGlu_n$ that is due to repression of de novo purine biosynthesis in bacteria cultured in medium containing purines should cause significant inhibition of the dehydrogenase but that this inhibition would be insufficient to prevent some accumulation of 10-formyl-$H_4PteGlu_n$ (15). The results obtained in the present study support this conclusion.

Under the described culture conditions, *L. casei* and *S. faecalis* require 5,10-methylene-$H_4PteGlu_n$ for the synthesis of thymidylate and ribothymine in $tRNA_f^{met}$ and 10-formyl-$H_4PteGlu_n$ for the biosynthesis of purines and formylmethionine. The reduced growth rate of *S. faecalis* in medium containing adenine proba-

bly resulted from the low proportion of folate present as $H_4PteGlu_n$ plus 5,10-methylene-$H_4PteGlu_n$ in these cells.

Although our data suggest that changes in the folylpolyglutamate distribution in bacteria are a secondary effect due to primary regulation of the one-carbon flux through pathways catalyzed by various folate-dependent enzymes, it may well be that these changes in the folate chain lengths could significantly affect the metabolic flux through some of the cycles of one-carbon metabolism and could play an important role in the regulation of one-carbon metabolism.

ACKNOWLEDGMENTS

We thank Jim Stoll and Alan Waldman for assistance in some of these studies, Jesse Rabinowitz (University of California at Berkeley) for the gift of purified *Clostridium* 10-formyl-$H_4PteGlu$ synthetase, and Gary Henderson (Scripps Clinic and Research Foundation, La Jolla, Calif.) and Roy Kisliuk (Tufts Medical School, Boston, Mass.) for gifts of methotrexate-resistant *L. casei* strains.

This work was supported by Public Health Service grant CA 22717 from the National Cancer Institute.

LITERATURE CITED

1. **Baggott, J. E., and C. L. Krumdieck.** 1979. Folate poly-γ-glutamyl derivatives as cosubstrates for chicken liver AICAR transformylase, p. 347–351. *In* R. L. Kisliuk and G. M. Brown (ed.), Chemistry and biology of pteridines. Elsevier/North-Holland Publishing Co., New York.
2. **Baugh, C. M., E. Braverman, and M. G. Nair.** 1974. The identification of poly-γ-glutamyl chain lengths in bacterial folates. Biochemistry **13**:4952–4957.
3. **Baugh, C. M., E. B. Braverman, and R. K. Sharma.** 1976. Studies on the metabolism of poly-γ-glutamates of folic acid in *E. coli* B. Fed. Proc. **35**:1543.
4. **Baugh, C. M., J. C. Stevens, and C. L. Krumdieck.** 1970. Studies on γ-glutamyl carboxypeptidase. 1. The solid phase synthesis of analogs of polyglutamates of folic acid and their effects on human γ-glutamyl carboxypeptidase. Biochim. Biophys. Acta **212**:116–125.
5. **Blakley, R. L.** 1969. The biochemistry of folic acid and related pteridines, p. 92–94. *In* A. Neuberger and E. L. Tatum (ed.), Frontiers of biology, vol. 13. North-Holland Publishing Co., Amsterdam.
6. **Blakley, R. L.** 1969. The biochemistry of folic acid and related pteridines, p. 219–358. *In* A. Neuberger and E. L. Tatum (ed.), Frontiers of biology, vol. 13. North-Holland Publishing Co., Amsterdam.
7. **Brody, T., B. Shane, and E. L. R. Stokstad.** 1979. Separation and identification of pteroylpolyglutamates by polyacrylamide gel chromatography. Anal. Biochem. **92**:501–509.
8. **Buehring, K. U., T. Tamura, and E. L. R. Stokstad.** 1974. Folate coenzymes of *Lactobacillus casei* and *Streptococcus faecalis*. J. Biol. Chem. **249**:1081–1089.
9. **Cheng, F. W., B. Shane, and E. L. R. Stokstad.** 1975. Pentaglutamate derivatives of folate as substrates for rat liver tetrahydropteroylglutamate methyltransferase and 5,10-methylenetetrahydrofolate reductase. Can. J. Biochem. **53**:1020–1027.
10. **Cichowicz, D. J., S. K. Foo, and B. Shane.** 1981. Folylpoly-γ-glutamate synthesis by bacteria and mammalian cells. Mol. Cell. Biochem. **39**:209–228.
11. **Covey, J. M.** 1980. Polyglutamate derivatives of folic acid coenzymes and methotrexate. Life Sci. **26**:665–678.
12. **Dev, I. K., and R. J. Harvey.** 1982. Sources of one-carbon units in the folate pathway of *Escherichia coli*. J. Biol. Chem. **257**:1980–1986.
13. **Foo, S. K., D. J. Cichowicz, and B. Shane.** 1980. Cleavage of naturally occurring folates to unsubstituted p-aminobenzoylpolyglutamates. Anal. Biochem. **107**:109–115.
14. **Furness, R. A. H., and P. C. Loewen.** 1981. Detection of p-aminobenzoylpoly-γ-glutamates using fluorescamine. Anal. Biochem. **117**:126–135.
15. **Harvey, R. J., and I. K. Dev.** 1975. Regulation in the folate pathway of *Escherichia coli*, p. 99–124. *In* G. Weber (ed.), Advances in enzyme regulation, vol. 13. Pergamon Press, Inc., Elmsford, N.Y.
16. **Kisliuk, R. L.** 1981. Pteroylpolyglutamates. Mol. Cell. Biochem. **39**:331–345.
17. **Kisliuk, R. L., Y. Gaumont, E. Lafer, C. M. Baugh, and J. A. Montgomery.** 1981. Polyglutamyl derivatives of tetrahydrofolate as substrates for *Lactobacillus casei* thymidylate synthetase. Biochemistry **20**:929–934.
18. **Krumdieck, C. L., P. E. Cornwall, R. W. Thompson, and W. E. White, Jr.** 1977. Studies on the biological role of folic acid polyglutamates, p. 25–42. *In* Food and Nutrition Board (ed.), Folic acid. Biochemistry and physiology in relation to the human nutrition requirement. National Academy of Science, Washington, D.C.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **Mansouri, A., J. B. Decter, and R. Silber.** 1972. Studies on the regulation of one-carbon metabolism. II. Repression-derepression of serine hydroxymethyltransferase by methionine in *Escherichia coli* 113-3. J. Biol. Chem. **247**:348–352.
21. **McBurney, M. W., and G. F. Whitmore.** 1974. Isolation and biochemical characterization of folate deficient mutants of Chinese hamster ovary cells. Cell **2**:173–182.
22. **McGuire, J. J., and J. R. Bertino.** 1981. Enzymatic synthesis and functions of folylpolyglutamates. Mol. Cell. Biochem. **38**:19–48.
23. **McGuire, J. J., P. Hsieh, J. K. Coward, and J. R. Bertino.** 1980. Enzymatic synthesis of folylpolyglutamates. Characterization of the reaction and its products. J. Biol. Chem. **255**:5776–5788.
24. **Miller, B. A., and E. B. Newman.** 1974. Control of serine transhydroxymethylase synthesis in *Escherichia coli* K12. Can. J. Microbiol. **20**:41–47.
25. **Ohara, O., and R. Silber.** 1969. Studies on the regulation of one-carbon metabolism. The effects of folate concentration in the growth medium on the activity of three folate-dependent enzymes in *Lactobacillus casei*. J. Biol. Chem. **244**:1988–1993.
26. **Scott, J. M., and D. G. Weir.** 1976. Folate composition, synthesis and function in natural materials. Clin. Haematol. **5**:547–568.
27. **Shane, B.** 1980. Pteroylpolyglutamate synthesis by *Corynebacterium species*. I. *In vivo* synthesis of folates. J. Biol. Chem. **255**:5649–5654.
28. **Shane, B.** 1980. Pteroylpolyglutamate synthesis by *Corynebacterium species*. II. Purification and properties of folylpolyglutamate synthetase. J. Biol. Chem. **255**:5655–5662.
29. **Shane, B.** 1982. High performance liquid chromatography of folates: identification of poly-γ-glutamate chain lengths of labeled and unlabeled folates. Am. J. Clin. Nutr. **35**:599–608.
30. **Shane, B., and E. L. R. Stokstad.** 1975. Transport and metabolism of folates by bacteria. J. Biol. Chem. **250**:2243–2253.
31. **Shane, B., and E. L. R. Stokstad.** 1976. Transport and utilization of methyltetrahydrofolates by *Lactobacillus casei*. J. Biol. Chem. **251**:3405–3410.
32. **Shane, B., and E. L. R. Stokstad.** 1977. Metabolism of 5-methyltetrahydrofolate by *Lactobacillus casei*. J. Gen. Microbiol. **103**:249–259.
33. **Shane, B., and E. L. R. Stokstad.** 1977. Rate limiting steps in folate metabolism by *Lactobacillus casei*. J. Gen.

Microbiol. **103**:261–270.

34. **Taylor, R. T., and M. L. Hanna.** 1977. Folate-dependent enzymes in cultured Chinese hamster cells: folylpolyglutamate synthetase and its absence in mutants auxotrophic for glycine + adenosine + thymidine. Arch. Biochem. Biophys. **181**:331–344.
35. **Whiteley, J. M., G. B. Henderson, A. Russell, P. Singh, and E. M. Zevely.** 1977. The isolation of dihydrofolate reductases by affinity chromatography on folate sepharose. Anal. Biochem. **79**:42–51.