The *trp* RNA-binding attenuation protein (TRAP) regulates the steady-state levels of transcripts of the *Bacillus subtilis* folate operon

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The *Bacillus subtilis* folate operon contains nine genes. The first six genes are involved in the biosynthesis of folic acid and tryptophan and have been characterized previously. The 3'-region of the folate operon contains three additional ORFs: *orf3*, potentially encoding a DNA-binding protein of 68 amino acids, *orf4*, encoding a protein of 338 amino acids with homology to the Orf1 of the *E. coli* *fis* operon, and a putative lysyl-tRNA synthetase gene (*lysS*). Four transcripts were identified which encode the first two, eight or all nine proteins or only the last protein LysS. The folate operon contains two promoters, one upstream of the first gene and the second preceding *lysS*. Transcription of the entire folate operon starts 33 bp upstream of the ATG codon of *pab*, the first gene of the operon. The *mtrB*-encoded *trp* RNA-binding attenuation protein (TRAP) dramatically reduces the steady-state levels of the folate operon transcripts encoding the first eight and all nine proteins, but only has a relatively small effect on the steady-state level of the 2.1 kb transcript encoding the first two genes of the operon, *pab* and *trpG*. In addition, transcription of the folate operon is regulated in a growth-phase-dependent manner. Transcripts were present in very low levels after mid-exponential phase, but were dramatically increased directly after transfer of the cells to fresh medium. These results indicate that transcription of the folate operon is regulated by TRAP and also depends on the growth phase of the culture.

Keywords: folate operon, regulation, *Bacillus subtilis*, transcription

INTRODUCTION

Folate derivatives are involved as cofactors in the biosynthesis of compounds such as purines, pyrimidines, glycine, methionine, choline and panthotenic acid. Folate derivatives are mainly needed during cell growth when DNA replication and RNA and protein synthesis are required and are not essential for non-growing cells. Biosynthesis of sufficient quantities of dihydrofolate is essential for cell growth and viability. Most bacterial species synthesize their own folate derivatives using GTP, chorismate and glutamine as precursors (Fig. 1a). The enzymic steps involved have been extensively studied in enteric bacteria (reviewed by Brown &

Williamson, 1982). More recently, *Bacillus subtilis* genes encoding enzymes involved in dihydrofolate biosynthesis were isolated and characterized. These genes were found in three different positions on the chromosome. The *mtr* operon (map position 204°) encodes the GTP cyclohydrolase I, and TRAP (tryptophan RNA-binding attenuation protein) (Babitzke *et al.*, 1992; Otridge & Gollnick, 1993). The folate biosynthetic operon (Fig. 1b) (Slock *et al.*, 1990) (map position 10°) contains *pab*, *trpG*, *pabC*, *sul* and two ORFs (*orf1* and *orf2*) probably encoding the dihydroneopterin aldolase (re-named *folA*) and the hydroxymethyl-dihydropterin pyrophosphokinase (re-named *folK*), respectively (Lacks *et al.*, 1995; Lopez & Lacks, 1993). In addition, the gene that may encode dihydrofolate synthetase (*folC*) is located at 246° on the *B. subtilis* map (Margolis *et al.*, 1993).

Abbreviation: TRAP, *trp* RNA-binding attenuation protein.

(a)

(b)

Fig. 1. Folate biosynthetic pathway and folate operon of *B. subtilis*. (a) Dihydrofolate biosynthetic pathway. The genes present in the folate operon (see part b) are shown in hatched boxes. *mtrB* and *folC* are located at two different positions in the *B. subtilis* genome. Genes involved in the specific dephosphorylation of dihydroneopterin triphosphate and dihydroneopterin monophosphate have not been identified. (b) Gene organization of the folate operon. *orf3*, *orf4* and *lysS* were identified by DNA sequence analysis (Ogasawara *et al.*, 1994; EMBL accession no. D26185, and our data). *cysK* and *rrnJ*, which encode cysteine synthetase A and rRNAs, respectively, do not belong to the folate operon. The functions of the first six genes, which are involved in dihydrofolate biosynthesis, are shown in (a), hatched boxes. The direction of transcription is from left to right. The coding sequences are shown by black arrows, the numbers indicate the position of the A of the ATG codon and the last base of the stop codon. Hatched boxes represent long intergenic regions, while the stem-loop motifs represent putative transcription terminators. Relevant recombinant plasmids containing restriction fragments of part of the operon are indicated below the *EcoRI* restriction map.

Thus, genes for most of the enzymes of folate biosynthesis are currently available. However it is still unclear whether specialized enzymes exist for the dephosphorylation of dihydroneopterin-triphosphate and dihydroneopterin-monophosphate in the GTP branch of the pathway and the genes involved have not been identified (Fig. 1a). Alternatively, these reactions may be catalysed by dephosphorylating enzymes with a broad substrate specificity or even by chemical dephosphorylation not requiring enzyme activity. We have previously shown that the pyrophosphohydrolase reaction could be efficiently catalysed *in vitro* in the absence of any enzyme by physiologically relevant concentrations of divalent cations (de Saizieu *et al.*, 1995).

The biosynthetic pathways for dihydrofolate and tryptophan are strongly interconnected. Both pathways use chorismate and glutamine to synthesize the *p*-aminobenzoic acid and *o*-aminobenzoic acid precursors of dihydrofolate and tryptophan, respectively (Kane *et al.*, 1972). Secondly, the TrpG protein (Fig. 1a) participates in the biosynthesis of *p*-aminobenzoic acid and *o*-aminobenzoic acid by the Pab-TrpG and TrpE-TrpG complexes, respectively (Goncharoff & Nichols, 1984). Finally, the regulatory TRAP negatively affects both biosynthetic pathways upon activation by L-tryptophan. Expression of the *trp* operon is regulated by a transcription attenuation mechanism: TRAP binding to the

leader sequence of the *trp* mRNA results in formation of a transcription termination stem-loop (Babitzke & Yanofsky, 1993; Kuroda *et al.*, 1988; Otridge & Gollnick, 1993). In addition, translation of *trpG*, which is located in the folate operon, is inhibited by L-tryptophan-activated TRAP, whereas transcription is not (Yang *et al.*, 1995). TRAP binds to a target sequence overlapping the RBS of *trpG* (Babitzke *et al.*, 1994). Thus, L-tryptophan not only affects its own synthesis, but via TrpG also the biosynthesis of dihydrofolate.

Here we describe the transcriptional organization of the folate operon and the growth-phase-dependent regulation of the encoded mRNAs. We also demonstrate a novel regulatory function for TRAP: it negatively affects the steady-state levels of the longer transcripts encoding all proteins of the folate operon.

METHODS

Bacterial strains, plasmids and media. Unless stated differently, *E. coli* XL1-Blue was used as host strain for all the cloning steps in *Escherichia coli*. The wild-type *B. subtilis* strain 1012 was obtained from the Bacillus Genetic Stock Center. All relevant strains and plasmids are listed in Table 1. Plasmid pK0101 (McDonald & Burke, 1982) containing the 4.9 kb fragment encoding part of the folate operon was kindly provided by Dr J. Slock. Further details on the construction of novel plasmids are given below. LB medium (Difco) was used as a rich culture medium for both *E. coli* and *B. subtilis*. *E. coli*

Table 1. Bacterial strains and plasmids

Strain/plasmid	Description or genotype	Reference/source
Strains		
<i>E. coli</i>		
XL1-Blue MRF'		Stratagene
M15[pREP4]	<i>lac, ara, gal, mtl</i>	Stueber <i>et al.</i> (1990)
<i>B. subtilis</i>		
1012	<i>leuA8 metB5 rM mM⁺</i>	Saito <i>et al.</i> (1979)
IBC72	<i>leuA8 metB5 rM mM⁺ mtrB::Cm^r</i>	Yang <i>et al.</i> (1995)
IO3C2	<i>leuA8 metB5 rM mM⁺ orf3::Cm^r</i>	This study
IO4C7	<i>leuA8 metB5 rM mM⁺ orf4::Cm^r</i>	This study
ZB493	<i>trpC2 pheA1 abrB707 SPβ c2 del2::Tn917::pSK10Δ6</i>	Zuber <i>et al.</i> (1987)
ZAS12	<i>trpC2 pheA1 abrB707 SPβ c2 del2::Tn917::pZAS12</i>	This study
ZAS32	<i>trpC2 pheA1 abrB707 SPβ c2 del2::Tn917::pZAS32</i>	This study
ZAS52	<i>trpC2 pheA1 abrB707 SPβ c2 del2::Tn917::pZAS52</i>	This study
ZAS52b	<i>trpC2 pheA1 abrB707 SPβ c2 del2::Tn917::pZAS52b</i>	This study
ZAS74	<i>trpC2 pheA1 abrB707 SPβ c2 del2::Tn917::pZAS74</i>	This study
ZAS96	<i>trpC2 pheA1 abrB707 SPβ c2 del2::Tn917::pZAS96</i>	This study
ZAS118	<i>trpC2 pheA1 abrB707 SPβ c2 del2::Tn917::pZAS118</i>	This study
ZAS1310	<i>trpC2 pheA1 abrB707 SPβ c2 del2::Tn917::pZAS1310</i>	This study
ZASLO	<i>trpC2 pheA1 abrB707 SPβ c2 del2::Tn917::pZAZLO</i>	This study
12A12	<i>leuA8 metB5 rM mM⁺ SPβ c2 del2::Tn917::pZAZ12</i>	This study
12A32	<i>leuA8 metB5 rM mM⁺ SPβ c2 del2::Tn917::pZAZ32</i>	This study
12A52	<i>leuA8 metB5 rM mM⁺ SPβ c2 del2::Tn917::pZAZ52</i>	This study
12A52b	<i>leuA8 metB5 rM mM⁺ SPβ c2 del2::Tn917::pZAZ52b</i>	This study
12A74	<i>leuA8 metB5 rM mM⁺ SPβ c2 del2::Tn917::pZAZ74</i>	This study
12A96	<i>leuA8 metB5 rM mM⁺ SPβ c2 del2::Tn917::pZAZ96</i>	This study
12A118	<i>leuA8 metB5 rM mM⁺ SPβ c2 del2::Tn917::pZAZ118</i>	This study
12A1310	<i>leuA8 metB5 rM mM⁺ SPβ c2 del2::Tn917::pZAZ1310</i>	This study
12ALO	<i>leuA8 metB5 rM mM⁺ SPβ c2 del2::Tn917::pZAZLO</i>	This study
Plasmids		
pK0101	Km ^r <i>pab⁺ trpG⁺ pabC⁺ Sul^r orf1⁺</i>	McDonald <i>et al.</i> (1982)
pZΔ327	Ap ^r Cm ^r . Used to construct <i>lacZ</i> fusions in <i>E. coli</i>	Zuber <i>et al.</i> (1987)
pZASn	pZΔ327 containing folate operon fragments in the <i>Hind</i> III site. See Fig. 2 for inserts.	This study
pFAA72	Ap ^r . 1 kb <i>Alu</i> I fragment of the 3'-end of <i>orf2</i> through the 3'-end of <i>orf4</i> in pBluescript	This study
pFAE103	Ap ^r . 1.9 kb <i>Eco</i> RI fragment of the 5'-end of <i>orf4</i> through the 3'-end of <i>lysS</i> in pBluescript	This study
pFAP157	Ap ^r . 3 kb <i>Pst</i> I fragment from the middle of <i>orf4</i> to the middle of <i>rrnJ</i> in pBluescript	This study
pIO3	Ap ^r Cm ^r . Integration vector used for <i>orf3</i> disruption	This study
pIO4	Ap ^r Cm ^r . Integration vector used for <i>orf4</i> disruption	This study
pFP2A	Ap ^r Cm ^r . Integration vector used for <i>mtrB</i> disruption	This study
pBEST401	Ap ^r Cm ^r . Plasmid containing the Cm ^r cassette	Itaya <i>et al.</i> (1990)

cells containing pUC-based plasmids were grown in the presence of 100 µg ampicillin ml⁻¹, while transformed *B. subtilis* strains were cultivated using 10 µg chloramphenicol ml⁻¹ or 5 µg neomycin ml⁻¹. Transformation of *E. coli* was carried out as described by Sambrook *et al.* (1989). *B. subtilis* was transformed as described by Piggot *et al.* (1984).

Isolation of the 3'-region of the folate operon. Plasmid pK0101 contained an additional 300 bp *Eco*RI restriction fragment (nucleotides 4601–4901) at the 3'-end of the known insert. This DNA fragment, which was not mentioned previously (Slock *et al.*, 1990), occurred adjacent to the

4880 bp *Eco*RI restriction fragment in the *B. subtilis* chromosome (Fig. 1). This DNA fragment was used as a probe for restriction mapping and chromosomal walking towards the 3'-end of the folate operon. Total *B. subtilis* genomic DNA was digested with various restriction enzymes, and Southern blots were performed using the 300 bp *Eco*RI restriction fragment as radiolabelled probe. A hybridizing region of 1.0 kb was seen upon *Alu*I digestion. This DNA fragment was isolated from an agarose gel and ligated into pBluescript SK(–) (Stratagene) linearized by *Eco*RV. *E. coli* transformants containing this 1.0 kb DNA fragment were isolated

by hybridization using the 300 bp *EcoRI* probe. The DNA sequence of this restriction fragment was determined using recombinant plasmid pFAA72 (Fig. 1b) by the dideoxy DNA sequencing procedure. Similarly, a 1.9 kb *EcoRI* restriction fragment (in pFAE103, Fig. 1b) and a 3 kb *PstI* restriction fragment (in pFAP157, Fig. 1b) were isolated and their DNA sequences determined.

Insertional gene inactivation. The *mtrB* gene was disrupted in strain IBC72 as described by Yang *et al.* (1995). Two different plasmid constructs were made to disrupt the *orf3* (pIO3, Table 1), and *orf4* (pIO4, Table 1) genes. Each plasmid contains approximately 550 bp of the respective sequence on each side of a chloramphenicol resistance marker to ensure efficient integration into the corresponding gene in the *B. subtilis* genome. A *HindIII* and an *EcoRI* site were introduced by PCR into the coding sequences of *orf3* and *mtrB*, respectively. In the case of *orf3*, the *HindIII* site was introduced at position 4671, which corresponds to the turn in the putative helix–turn–helix motif. The existing *PstI* restriction site at position 5187 was used for *orf4* disruption. In addition, a stop codon in the correct reading frame was introduced in each case to ensure that translation terminated at that point. These DNA fragments were cloned into pBluescript SK(–) following the same procedure as described by Yang *et al.* (1995). The chloramphenicol-resistance cassette from pBEST401 (Table 1) was then inserted into the above-mentioned restriction sites. Ten micrograms of each plasmid linearized with *ScaI* was used for *B. subtilis* transformation. Chloramphenicol-resistant transformants having the replacement of the wild-type gene by the interrupted DNA sequence were identified using PCR or Southern blot analysis. All mutants obtained resulted from a double cross-over and did not contain any vector sequences. The resulting strains were named IO3C2 and IO4C7 for disruption of *orf3* and *orf4*, respectively (Table 1).

***lacZ* fusions.** Transcriptional fusions to the *E. coli lacZ* gene were constructed to determine promoter activity. PCR generated fragments (1–1.4 kb) of the folate operon containing *HindIII* sites at both ends were inserted into the *HindIII* site of plasmid pZΔ327 (Zuber & Losick, 1983) upstream of a promoterless *E. coli lacZ* gene containing a *B. subtilis* RBS. These constructs were then linearized with *BalI* and 10 µg plasmid DNA was transformed into *B. subtilis* strain ZB493 (Table 1, ZAS*n* strains, *n* representing the laboratory number of the strain containing a specific plasmid integrated into the genome). This host strain allows the use of the specialized transducing bacteriophage SPβ to transfer the fusion to other strains (Zuber & Losick, 1983). As strain ZB493 is *abrB* and *trp* (Strauch *et al.*, 1989), all the constructs were subsequently transduced into the wild-type strain 1012 (Table 1, 12A*n* strains, *n* representing the laboratory number of the strain containing a specific ZAS*n* plasmid integrated into the genome) to avoid any possible secondary effect due to these mutations. β-Galactosidase activity was determined by a protocol based on that of Turner *et al.* (1994). The specific activities are given in Miller units (Miller, 1972). Three independent clones were analysed in each case. Very similar β-galactosidase activities were obtained with the ZB493- and 1012-derived strains, indicating that *abrB* and *trp* mutations of ZB493 do not affect the activity of the folate operon promoters.

Northern blot analysis. *B. subtilis* cells were collected by centrifugation and stored frozen in liquid nitrogen. Unless mentioned, cells were harvested at OD₆₀₀ ~ 0.8 (spectrophotometer). RNA was prepared using a phenol grinding method (Maes & Messens, 1992), except that the RNA obtained was subjected to an additional DNaseI treatment

followed by a phenol extraction and ethanol precipitation. The RNA pellet was then dissolved in H₂O and stored at –70 °C. We obtained about 1 mg total RNA from a 50 ml culture grown to OD₆₀₀ 0.8. Total cellular RNA (15 to 40 µg per lane) was electrophoresed on a formaldehyde gel and transferred to a nylon filter (Gene-Screen). RNA size standards were purchased from Gibco. [α-³²P]dCTP-labelled DNA probes were obtained by random priming of PCR-generated DNA fragments (Fig. 4a). Probe A covers the 5' region of the *pab* gene; probe B, *orf4*, and probe D, the middle part of the *lysS* gene (Fig. 4a).

Primer-extension experiments. RNA was isolated from *B. subtilis* IBC72 (*mtrB*[–]) grown to an OD₆₀₀ of 0.8. Primer extension experiments were performed as previously described (Nakano *et al.*, 1991) using 35 µg total RNA per reaction and oligonucleotide Prun2 (Fig. 3a). A DNA sequence ladder obtained using the dideoxy-chain termination method and the same primer was used to determine the exact transcription start position.

RNase A/T1 mapping. RNase mapping experiments were carried out as described (Melton *et al.*, 1984). Depending on the experiment, 10 to 40 µg of total RNA from the 1012 and IBC72 strains and 40000 c.p.m. of a gel-purified RNA antisense probe (molar excess compared to the specific folate mRNA) were mixed and dried in a speed-vac. The RNA probe was labelled by *in vitro* transcription from a T3 promoter in the presence of [³²P]UTP. The dried pellets were redissolved in 20 µl hybridization solution (40 mM PIPES pH 6.7, 80% formamide, 0.4 M NaCl and 1 mM EDTA), heated to 90 °C for 5 min and then incubated overnight at 50 °C. Then 100 µl RNase A/T1 mix [10 mM Tris pH 7.5, 5 mM EDTA, 0.3 M NaCl containing 80 µg RNase A ml^{–1} (Boehringer) and 50 U RNase T1 ml^{–1} (Boehringer)] were added and incubation continued for 40 min at 26 °C. One µl of 20% SDS and 3 µl Proteinase K (10 mg ml^{–1}) were added and incubated for 15 min at 37 °C. The mix was extracted with phenol/chloroform, 20 µg carrier (glycogen) was added and the RNA was ethanol-precipitated. The pellet was dissolved in formamide-containing gel loading buffer and run on a 6% sequencing gel. pBR322 digested with *HinfI* and filled in with Klenow polymerase was used as molecular size marker. In later experiments, an RNase mapping kit (Ambion) was used and similar results were obtained. The amount of protected RNA present in the individual bands was measured using the quantification program of the Molecular Dynamics Phosphor-imager. These values were corrected to give the relative amount of folate mRNA per cell. This was done by dividing the total RNA yield per sample by the total number of cells used to prepare the RNA. Cell numbers were determined using the OD₆₀₀ measurement (1 OD unit corresponding to 10⁸ cells). For this calculation, we assume that the cell size remains unchanged. The amount of total RNA used in the RNase protection experiments was kept constant. Drastically different amounts of total RNA were extracted at different time points. At *t* = 0, 18 µg per 10⁸ cells; at *t* = 15 min, 27 µg per 10⁸ cells; at *t* = 1 h, 115 µg per 10⁸ cells; at *t* = 2 h, 475 µg per 10⁸ cells and at *t* = 4 h, 76 µg per 10⁸ cells. A similar drastic increase in the cellular content of total RNA upon onset of growth was described for *E. coli* (Gausing, 1977).

Phospho-imager processing of data. All pictures of the Northern blot, primer extension and RNase mapping experiments shown in this paper were generated using a Molecular Dynamics Phospho-imager.

RESULTS

Identification of three new ORFs at the 3'-end of the folate operon

The portion of the folate operon characterized by Slock *et al.* (1990) (up to the *EcoRI* restriction site in *orf2*, position 4601 in Fig. 1b) contains six ORFs [*pab*, *trpG*, *pabC*, *sul*, *folA* (previously called *orf1*)] and *folK* (previously called *orf2*) encoding proteins involved in the biosynthesis of dihydrofolate (Fig. 1a). We isolated and characterized a 3030 bp DNA fragment containing the end of *folK* and three additional ORFs belonging to the folate operon (Fig. 1 and below). The DNA sequence obtained was 100% identical to that determined by Ogasawara *et al.* (1994; EMBL accession number D26185).

The stop codon of *folK* was found at position 4619. The *folK* gene product is predicted to contain 167 amino acids with a theoretical molecular mass of 19 kDa. The first 162 amino acids were 63% identical to the *E. coli* HPPK (6-hydroxymethyl-7,8 dihydroneopterin pyrophosphokinase) (Lopez & Lacks, 1993).

orf3 and *folK* show an overlap of 49 nucleotides (Fig. 1b). *orf3* is preceded by a sequence consensus (AAGAAGG) resembling a *B. subtilis* RBS. The coding sequence starts at position 4571 and ends at 4780. The putative *orf3* gene product is 68 amino acids long and shows 29% to 35% identity with several DNA-binding proteins from *B. subtilis* (SinR, Gaur *et al.*, 1991; ImmF, van Kaer *et al.*, 1987) and *E. coli* (DicA, Béjar *et al.*, 1986). Orf3 may be a DNA-binding protein since it has a putative helix–turn–helix motif with conserved hydrophobic residues which are found in DNA-binding proteins. Interruption of the coding sequences of *orf3* or *orf4* (see below) by an antibiotic-resistance gene did not affect the levels of folate operon transcripts nor the growth-phase-dependent regulation of transcripts of the folate operon (not shown).

orf3 and *orf4* are separated by an intergenic region of five nucleotides (Fig. 1a). *orf4* starts at position 4786 and ends at 5805. It encodes a putative protein of 338 amino acids with a predicted molecular mass of 37.7 kDa. It is 40% identical to Orf1 in the *fis* operon from *E. coli* (Ninnemann *et al.*, 1992) and shows 38% identity to NtrB from *Rhizobium phaseoli* (Patriarca *et al.*, 1993). A very similar ORF was also found in *Rhodobacter capsulatus* (Foster-Hartnett *et al.*, 1993). The function of these proteins, which are always co-transcribed with DNA-binding proteins, is not known.

lysS and *orf4* are separated by a 91 bp intergenic region. *LysS* is 53% identical to the *E. coli* lysyl-tRNA synthetase enzyme (Nakamura & Ioto, 1993). Previous mapping experiments also located the *B. subtilis* *lysS* gene close to the *sul* gene (74% co-transformation; Racine & Steinberg, 1974). Two functional lysyl-tRNA synthetase genes were described in *E. coli* (Nakamura & Ioto, 1993). However, since we were unable to obtain any transformant when trying to disrupt the *lysS* gene in

B. subtilis, *lysS* may be the sole functional lysyl-tRNA synthetase in *B. subtilis*.

A putative stable hairpin structure possibly functioning as a strong transcription terminator was found between the *lysS* and the *rrnJ* (encoding 16S, 23S and 5S rRNAs). These results suggested that *lysS*, but not *rrnJ*, belongs to the folate operon. RNA mapping and Northern blot experiments (see below) confirmed this hypothesis.

The folate operon contains two promoters

Mapping of fragments with promoter activity was carried out using transcriptional fusions between PCR fragments of the folate operon (pZAS plasmids, Fig. 2) and the *lacZ* gene. These constructs were integrated as single-copy genes in the genome of *B. subtilis* strains ZB493 and 1012 (Table 1). Two separate regions of the folate operon showed promoter activity. The first region is located between *cysK* and *pab*: ZAS12 and ZAS32, but not ZAS52 (Fig. 2), resulted in expression of the *lacZ* gene. The second region is located in ZASL0 and not within ZAS1310, indicating that the *lysS* gene possesses its own promoter. The promoter activities deduced from the β -galactosidase expression levels were unexpectedly low, especially since in strain 1012 a 2.1 kb transcript encoding *pab* and *trpG* and a 1.5 kb transcript for *lysS* were detected by Northern blotting experiments (see below). The reasons for the low expression levels are unclear. It could indicate that folate operon sequences necessary for higher level expression of the reporter protein are lacking.

Primer-extension experiments located the transcription start of the folate operon 33 nucleotides upstream of the *pab* ATG codon (Fig. 3a, arrowhead and Fig. 3b, lane 1). The –10 and –35 regions cannot be assigned without ambiguity. Two possible –10 regions, TGCTTTACA and CTTTACAAT (Fig. 3a), were seen, both showing a five out of eight match to the 'extended –10' class of bacterial promoters (TGNTATAAT) (Belyaeva *et al.*, 1993; Helmann, 1995). The –35 sequence could be TTCACT or TTTCA. In each case, a non optimal –10/–35 spacing is obtained and this could account for weak promoter activity seen with *pab*–*lacZ* fusions. The degenerate palindromic sequence observed between positions –96 and –77 (see boxes Fig. 3a) is not essential for the low level of folate promoter activity observed here, since this sequence was included in ZAS12 *lacZ* fusion but not in ZAS32, and both clones gave similar β -galactosidase activities (Fig. 2).

Inactivation of the *mtrB*-encoded TRAP results in dramatically increased levels of full-sized transcripts of the folate operon

Northern blot experiments were performed to analyse the transcripts of the folate operon. With RNA from the wild-type *B. subtilis* 1012 strain grown to an OD₆₀₀ of 0.8, only a 2.1 kb and a 0.8 kb band were seen which hybridized to probe A covering *pab* (Fig. 4a and Fig. 4b, lane 1) and to DNA fragments corresponding to the

Fig. 2. Functional mapping of promoter sequences. Different regions (black bars) of the folate operon were tested for promoter activity using transcriptional fusions to the *lacZ* reporter gene in plasmid pZΔ327. These plasmids were then integrated as single copy genes in the genome of *B. subtilis* strains ZB493 and 1012. β -Galactosidase activities (means from duplicate measurements with each of three independent clones) were measured in *B. subtilis* cells containing these fusions integrated into the chromosome as a single copy gene. Very similar results were obtained with both strains. Enzyme activities are indicated in Miller units above the corresponding fragments. The β -galactosidase background activity in cells containing only the control plasmid pZΔ327 varied between 0.2 and 0.4 U. Relevant restriction enzyme sites are shown: B, *Bam*I and H, *Hind*III.

trpG gene (not shown). The 2.1 kb mRNA is long enough to completely encode *pab* and *trpG* and may terminate at a putative transcription terminator located downstream of the *trpG* gene. The nature of the 0.8 kb band is currently unclear as its size is too short to contain the complete coding sequence of either gene. It may either represent a stable degradation product or, alternatively, result from non-specific hybridization. Background hybridization to 16S and 23S rRNA bands can also be seen. This is a common observation which has been attributed to trapping of the mRNA by the abundant ribosomal species (Simpson *et al.*, 1990). All attempts to visualize transcripts containing the 3'-part of the operon (starting with *pabC*) using Northern blots with RNA from wild-type *B. subtilis* cells gave unclear results with very faint signals even when as much as 40 μ g of total RNA was used (Fig. 4b, lane 1 and not shown). RNase protection assays, however, clearly showed that at least regions of such transcripts exist under the growth conditions used (see below).

Interruption of *mtrB* (encoding TRAP) resulted in detection of additional folate operon transcripts of 5.9 and 7.5 kb (Fig. 4, lanes 2, 3 and 5). The transcripts correspond to the entire operon (*pab* to *lysS*, 7.5 kb transcript) or *pab* to *orf4* (5.9 kb transcript) (Fig. 4a). Since promoter activity was found upstream of the *lysS* gene (Fig. 2), a transcript of 1.5 kb encoding *LysS* is expected. Although a 1.5 kb transcript was seen in wild-type 1012 cells, it is apparently absent in strain IBC72 which lacks a functional TRAP (compare Fig. 4b, lanes 4 and 5). Thus, TRAP encoded by *mtrB* may affect either transcription of the folate operon or the stability of the 5.9 and 7.5 kb transcripts.

The folate operon contains three transcription terminators

The Northern blot experiments indicated that three transcription terminators should be present in the folate operon, one after *trpG*, the second after *orf4* and the third after *lysS*. In addition, a transcription terminator should be present after *cysK*, the last gene of the preceding operon. RNase protection assays with RNA from strain 1012 were used to directly locate sites involved in transcription termination or initiation. The antisense RNA probes used (Fig. 5a) cover the regions containing the putative transcription terminators. As an internal control for the proper functioning of the RNase A/T1 digestion, each antisense probe contains a tail of sequences not complementary to folate operon transcripts.

The CP anti-sense RNA (covering the region between -279 and -7) contains the 3'-end of the *cysK* gene and most of the intergenic region between *cysK* and *pab*. The longest protected fragment of 180 nucleotides corresponds to the size expected for the 3'-end of the *cysK* mRNA terminating at the putative rho-independent terminator (positions -122 to -97). Additional shorter bands may have resulted from further degradation by the RNase A/T1 treatment of the 180 nucleotides fragment. A faint band of 270 bases corresponding to complete protection of the antisense fragment was seen upon longer exposure (not shown). Thus, transcription termination after *cysK* may be slightly leaky.

The antisense portion (268 nucleotides, positions 1393-1661) of the PT probe (Fig. 5a) was completely protected. Thus, no transcription termination or initiation occurs within this region, which covers the 3'-end of *pab* and the 5'-end of *trpG* (Fig. 5b, lanes 4-6). In contrast, two different protected nucleotide fragments were seen with the TP antisense RNA probe (positions 1992-2262), which covers the 3'-end of *trpG* and the 5'-end of *pabC* (Fig. 5a). The full-size protected fragment of 270 bases (Fig. 5b, lane 9) indicates transcription across the *trpG*-*pabC* junction. It, however, remains unclear why only very low amounts of the longer transcripts were detected in the Northern blot experiments. Possibly, the longer transcripts are unstable in wild-type 1012 cells and stable in the mutant lacking a functional TRAP. The presence of the additional shorter fragment of 115 bases (Fig. 5b, lane 9) is consistent with transcription termination at the 3'-end of *trpG*. This protected fragment may correspond to the 3'-end of the 2.1 kb *pab*-*trpG* transcript detected in the Northern blot experiments. Similarly, results obtained with the antisense probe covering the *orf4*-*lysS* intergenic region were consistent with partial transcription termination occurring in the intergenic region between *orf4* and *lysS* (not shown). A putative rho-independent terminator was indeed found between nucleotides 5828 and 5850.

The protected fragment of 220 bases observed with the LS antisense RNA (positions 7222-7576, Fig. 5a)

Fig. 3. The transcription start site at the 5'-end of the folate operon. (a) The DNA sequence upstream of the first gene of the folate operon (*pab*) and the start of *pab*. The ATG codon of *pab* is indicated (***). The transcription start was determined using primer extension experiments using the primer Prun2. The position of the observed transcription start (A) is indicated by ►. All putative -10 and -35 sequences are underlined. The position of a palindromic sequence is indicated by arrows above the boxed DNA sequence. (b) Phospho-imager picture of a primer-extension experiment. Dideoxy DNA sequence analysis (lanes T, G, C, A) and primer-extension reactions (lane 1 with added *B. subtilis* RNA and lane 2 without added RNA) were performed using the same primer, Prun2. The RNA used was isolated from exponentially growing IBC72 cells, which lack a functional TRAP.

indicates efficient transcription termination after the *lysS* gene (Fig. 5b lanes 10–12) at a putative rho-independent transcription terminator present between positions 7404–7437.

Thus, the folate operon starts with the *pab* gene and extends until the end of the *lysS* gene encoding a lysyl-tRNA synthetase. It has at least one internal promoter and, in addition to the transcription terminators after *cysK* and *lysS*, two internal sequences at which transcription termination can be detected.

Transcription of the folate operon is dramatically increased upon induction of cell growth

RNase protection experiments (Fig. 6) were used to quantify the levels of folate mRNAs in *B. subtilis* 1012 under various growth conditions. Low levels of folate mRNA were detected in cells grown beyond mid-exponential phase (Fig. 6b, samples obtained after growth for 4 h or overnight). However, higher levels of

Fig. 4. Northern blot hybridization experiments. (a) Schematic representation of the folate operon, the DNA fragments used as radiolabelled hybridization probes (A–C) and the locations of the transcripts observed. (b) Phospho-imager picture of a Northern blot hybridization. RNA was prepared from cells grown to early-exponential phase (OD_{600} 0.8). Total RNA from the wild-type *B. subtilis* 1012 (lanes 1 and 4) or the IBC72 mutant lacking a functional TRAP (lanes 2, 3 and 5) was subjected to gel electrophoresis and, after transfer to a filter, hybridized with the indicated radiolabelled DNA fragments [A–C, (a)]. Vertical bars indicate the positions of 23S and 16S rRNAs. The positions of hybridizing bands are shown by arrows. The nature of the 0.8 kb band is currently unclear (see Results). We assume that the accumulation of hybridizing material (*) at the front of the rRNA bands results from the use of 40 µg total RNA per lane and does not represent discrete folate operon transcripts.

mRNA as a fraction of total cellular RNA were seen between 15 min and at least 2 h after dilution of stationary-phase cells into fresh medium (Fig. 6b). Since the amount of total RNA per cell also increases dramatically upon initiation of growth, the effect is much more pronounced when the number of folate mRNA molecules per cell is determined. Quantification of the results obtained (see Methods) showed that the number of folate mRNAs per cell increased several thousandfold upon the onset of growth when compared to the amount found in stationary phase cells (Fig. 6c). The number of folate mRNA molecules per cell was maximal 2 h after dilution and decreased to the basal level between 2 and 4 h after the nutrient upshift (Fig. 6c and data not shown). As Northern blot

Fig. 5. Determination of sites of transcription termination. (a) Schematic representation of the folate operon and the positions of antisense RNA probes (horizontal black bars) used in RNase mapping experiments. The 32 P-labelled antisense RNAs were prepared by *in vitro* transcription using PCR fragments cloned into pBluescript. Each antisense RNA also contains some additional pBluescript vector sequences (non-horizontal black bars) as a control for the RNase A/T1 activity. Hatched areas correspond to longer intergenic regions. Stem-loops show the positions of putative rho-independent transcription terminators. (b) Phospho-imager pictures of RNase mapping experiments. Lanes P, full-length antisense RNA probe (including some pBluescript sequences). Lanes C, the antisense probe was incubated with yeast tRNA and subsequently treated with the RNase A/T1 mixture. This control shows possible protected fragments resulting from non-specific protection or secondary structures in the antisense RNA probe. Lanes S, 20 μ g total *B. subtilis* 1012 RNA was incubated with the respective antisense probe prior to treatment with the RNase A/T1 mixture. Arrowheads indicate the positions of the relevant protected antisense fragments. Lanes M, 32 P-labelled ssDNA marker.

antisense fragment since the transcripts also contain sequences from the multiple cloning site of the pBluescript vector. Lane 6 (left) and lanes 7 (middle and right), incubation of the radiolabelled antisense RNA with RNases, but without prior incubation with total RNA of *B. subtilis* strain 1012. Some of the protected fragments seen in lanes 2–6 of the right panel were also detected without incubation with *B. subtilis* 1012 RNA (lane 7). They, thus, are likely to reflect secondary structure within the antisense probe used. (c) Quantification of the data obtained, some of which are presented in (b) for the SO probe; $\circ$, d.p.m. in protected fragments per 10^8 cells is a measure of the relative mRNA levels per cell; $\square$, OD_{600} . The peak observed 2 h after shift mainly reflects a drastic increase in the total cellular content of RNA (see Methods for details).

Fig. 6. Levels of folate mRNA in various growth phases. (a) Schematic representation of the folate operon including the position of the antisense RNA fragments used (see also legend to Fig. 5). (b) Phospho-imager pictures of RNase mapping experiments using the indicated antisense RNAs. An overnight, stationary culture of wild-type *B. subtilis* strain 1012 (0 min) was used to inoculate fresh medium. Samples were taken at the indicated times after inoculation (15 min, 1, 2 and 4 h), total RNA was prepared and used in RNase protection experiments. In the different RNase protection experiment equal amounts of radiolabelled antisense RNA probe and of RNA *B. subtilis* strain 1012 were used and equivalent samples were loaded into each lane. Conditions were chosen such that the radiolabelled antisense RNA was in excess and that the amount of protected RNAs obtained could be used to quantify the relative folate mRNA levels in the various growth phases. Lanes: 1, radiolabelled antisense RNA fragments used; lanes 2–5 (left) and lanes 2–6 (other panels), RNase-protection using total RNA obtained at the indicated time points. The arrowheads show protected antisense RNA fragments corresponding to full protection of the entire antisense region of the probe. The protected fragments are shorter than the radiolabelled [legend continues at foot of left hand column]

experiments with mRNA from wild-type *B. subtilis* strain 1012 showed accumulation of a 2.1 kb transcript encoding *pab* and *trpG*, but failed to show discrete bands corresponding to longer transcripts encoding the 5'- and 3'-segments of the folate operon, this suggests that accumulating mRNAs covering other parts of the folate operon may be heterogeneous in size. Thus, transcription of the various regions of the folate operon appears to be affected similarly during growth.

DISCUSSION

TRAP negatively affects the steady-state levels of the 5.9 and 7.5 kb transcripts of the folate operon

In Northern blot experiments using RNA from wild-type *B. subtilis* 1012 cells, a 1.5 kb transcript encoding LysS and a 2.1 kb transcript encoding Pab and TrpG were detected, but no discrete longer transcripts encoding the other genes of the operon were found. In a *mtbB* mutant lacking a functional TRAP, two additional major transcripts of 5.9 kb, encoding Pab, TrpG, PabC, Sul, FolaA, FolK, Orf3 and Orf4, and of 7.5 kb, encoding LysS in addition to the other eight proteins, were seen. Thus, TRAP negatively affects the accumulation of both the 5.9 kb and the 7.5 kb mRNAs. Previously, we have shown that TRAP reduced threefold the steady-state levels of the 2.1 kb transcript encoding Pab and TrpG (Yang *et al.*, 1995). Thus, although the steady-state levels of at least three mRNAs encoded by the folate operon are reduced by TRAP, the effect is much more pronounced for the longest transcripts, which were not detected in wild-type cells. RNA protection experiments indicated that at least fragments of the longer transcripts are present in wild-type cells. Currently, several possibilities exist to explain these data, but the exact mechanism remains to be elucidated.

Firstly, TRAP could affect the half-life of the folate mRNAs by an unknown mechanism. TRAP specifically inhibits the translation of the TrpG protein by binding to the mRNA at a site overlapping the *trpG* RBS (Yang *et al.*, 1995). In addition to the effect on *trpG* translation, this could result in increased RNase-sensitivity of folate mRNAs, possibly because of decreased protection by ribosomes or because of changes in the secondary structures of the mRNAs. Alternatively, binding of TRAP to other parts of the folate mRNAs could decrease their half-life, or TRAP itself could be a folate-transcript-specific RNase.

Secondly, TRAP reduces the steady-state level of the 5.9 and 7.5 kb mRNAs by a partial transcription termination after the *trpG* gene. TRAP was previously shown to promote attenuation of the tryptophan operon (Babitzke & Yanofsky, 1993; Kuroda *et al.*, 1988; Otridge & Gollnick, 1993). The consensus sequence for TRAP binding consists of GAG and UAG repeats (up to 11) spaced by two to three nucleotides (Babitzke *et al.*, 1996). Although transcription termination occurs after the *trpG* gene in the folate operon and results in the 2.1 kb transcript, no evidence is available for regulation

of this transcription termination by TRAP. In addition, no homology to the consensus sequence described for the binding of TRAP (Babitzke *et al.*, 1994) was found in the immediate vicinity of the transcription terminator. TRAP could, however, indirectly affect transcription termination by regulation of the activity of an unknown transcription attenuator molecule or affect attenuation by binding to a site upstream of *trpG*.

The effects of TRAP on attenuation of the *trp* operon and translation of *trpG* are dependent on the presence of tryptophan (Babitzke *et al.*, 1994; Yang *et al.*, 1995). Whether the effect of TRAP on folate mRNA accumulation is also dependent on tryptophan remains to be established. The novel regulatory function of TRAP described here shows an additional link between the tryptophan and the folate biosynthetic pathways.

The *lysS* gene is encoded by two mRNA species

Two transcripts exist for the lysyl-tRNA synthetase gene, the 7.5 kb transcript encoding all genes of the folate operon and a 1.5 kb transcript encoding only the *lysS* gene. Interestingly, another gene of the folate biosynthetic pathway, the *folC* gene encoding the dihydrofolate synthetase, is co-transcribed with a tRNA synthetase gene, encoding valyl-tRNA synthetase (unpublished results). All known *B. subtilis* aminoacyl-tRNA synthetases (except for the methionyl- and the glutamyl-tRNA synthetases) are regulated by a common transcription attenuation mechanism (Grundy & Henkin, 1993). In cells containing high amounts of an amino acid and its charged tRNA, expression of the corresponding tRNA synthetase gene is repressed. The T-box mediating transcription termination by the charged tRNA was found preceding aminoacyl-tRNA synthetase genes, including the valyl-tRNA synthetase gene, but was not found in the promoter regions of the *lysS* gene and the folate operon. As was shown already for the genes encoding the methionyl- and the glutamyl-tRNA synthetases, expression of the *lysS* gene may be regulated differently. Although nothing is known about regulation of expression of the 1.5 kb *lysS* transcript, expression of the 7.5 kb transcript is controlled by TRAP and also shows a growth-phase-dependent regulation.

Growth-phase-dependent regulation

Stationary-phase cells contained only very low levels of folate mRNAs, while cells in early exponential phase had dramatically increased levels of folate operon transcripts. In other systems, addition of chloramphenicol to starved cells resulted in the abrupt disappearance of the stringent response molecule ppGpp due to the accumulation of amino acylated tRNAs resulting from inhibition of protein synthesis (Gallant, 1979). As a result, the activity of stringent response-controlled promoters is restored (Sokawa & Sokawa, 1978). The mechanisms controlling the growth-phase-dependent accumulation of transcripts of the folate

operon is not known. However, we observed that chloramphenicol treatment of stationary-phase cells also resulted in increased folate mRNA levels (unpublished results), suggesting a function of stringent response, and thus ppGpp, in the regulation of the folate operon promoter. *De novo* protein synthesis was also not required for the transcriptional activation of the folate promoter. In addition, TRAP may not play a direct role in growth-phase-dependent regulation of transcription of the folate operon as similar mRNA levels were measured in the wild-type 1012 and the 1012-*mtrB* mutant (unpublished results). We, thus, suggest that the growth-phase-dependent changes in expression of the folate operon are controlled by stringent response and do not involve TRAP.

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