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).
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 otherwise, *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. Stolz. 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*
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 EcoRI 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 EcoRI 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 EcoRI restriction fragment as radiolabelled probe. A hybridizing region of 1.0 kb was seen upon AluI digestion. This DNA fragment was isolated from an agarose gel and ligated into pBluescript SK(-) (Stratagene) linearized by EcoRV.

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description or genotype</th>
<th>Reference/source</th>
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<td><strong>Strains</strong></td>
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<tr>
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<td>Stratagene</td>
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<td><strong>Plasmids</strong></td>
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<td>Zubert et al. (1987)</td>
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<td>pFAE103</td>
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<td>pBEST401</td>
<td>Ap' Cm'. Plasmid containing the Cm' cassette</td>
<td>Itaya et al. (1990)</td>
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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 pFAEI03, 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 (pION, Table 1), and orf4 (pION, 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 I03C2 and I04C7 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 pZAS27 (Zuber & Losick, 1983) upstream of a promoterless *E. coli lacZ* gene containing a *B. subtilis* RBS. These constructs were then linearized with Ball and 10 μg plasmid DNA was transformed into *B. subtilis* strain ZB493 (Table 1, ZASn 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 *orf3* and *trp* (Straub et al., 1989), all the constructs were subsequently transduced into the wild-type strain 1012 (Table 1, 12An strains, n representing the laboratory number of the strain containing a specific ZASn 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 DNasel 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 HindIII and filled in with Klenow polymerase was used as molecular size marker. In later experiments, an RNase A/RNA 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 Phospho-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 10⁶ cells; at t = 15 min, 27 μg per 10⁶ cells; at t = 1 h, 115 μg per 10⁶ cells; at t = 2 h, 475 μg per 10⁶ cells and at t = 4 h, 76 μg per 10⁶ 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 dihydropterin 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, Bejar 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 DNA-binding proteins, is not known.

orf4 and folA 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 higher level expression of the reporter protein are lacking.

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.

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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
trpG gene (not shown). The 2.1 kb mRNA is long enough to completely encode \( \text{pab} \) and \( \text{trpG} \) and may terminate at a putative transcription terminator located downstream of the \( \text{trpG} \) 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 \( \text{pabC} \)) using Northern blots with RNA from wild-type \( B. \ subtilis \) cells gave unclear results with very faint signals even when as much as 40 \( \mu \)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 \( \text{mtrB} \) (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 (\( \text{pab} \) to \( \text{lysS} \), 7.5 kb transcript) or \( \text{pab} \) to \( \text{orf4} \) (5.9 kb transcript) (Fig. 4a). Since promoter activity was found upstream of the \( \text{lysS} \) gene (Fig. 2), a transcript of 1.5 kb encoding \( \text{LysS} \) 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 \( \text{mtrB} \) 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 \( \text{trpG} \), the second after \( \text{orf4} \) and the third after \( \text{lysS} \). In addition, a transcription terminator should be present after \( \text{cysK} \), 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 \( \text{cysK} \) gene and most of the intergenic region between \( \text{cysK} \) and \( \text{pab} \). The longest protected fragment of 180 nucleotides corresponds to the size expected for the 3'-end of the \( \text{cysK} \) 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 \( \text{cysK} \) 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 \( \text{pab} \) and the 5’-end of \( \text{trpG} \) (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 \( \text{trpG} \) and the 5'-end of \( \text{pabC} \) (Fig. 5a). The full-size protected fragment of 270 bases (Fig. 5b, lane 9) indicates transcription across the \( \text{trpG} \) to \( \text{pabC} \) 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 \( \text{trpG} \). This protected fragment may correspond to the 3'-end of the 2.1 kb \( \text{pab} \) to \( \text{trpG} \) transcript detected in the Northern blot experiments. Similarly, results obtained with the antisense probe covering the \( \text{orf4} \) to \( \text{lysS} \) intergenic region were consistent with partial transcription termination occurring in the intergenic region between \( \text{orf4} \) and \( \text{lysS} \) (not shown). A putative rho-independent terminator was indeed found between nucleotides 5828 and 3830.

The protected fragment of 220 bases observed with the LS antisense RNA (positions 7222–7576, Fig. 5a)
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**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 P. 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 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. 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 (OD₆₀₀ 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.
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 \textit{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 5, 20 pg total \textit{B. subtilis} strain 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 \textit{B. subtilis} strain 1012. Some of the protected fragments seen in lanes 2-6 of the right panel were also detected without incubation with \textit{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; ○, d.p.m. in protected fragments per $10^6$ cells is a measure of the relative mRNA levels per cell; [], 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 \textit{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 \textit{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 *mtrB* mutant lacking a functional TRAP, two additional major transcripts of 5.9 kb, encoding Pab, TrpG, PabC, Sul, FolA, FolK, Orf3 and Orf4, and 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
opener 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 opener promoter. De novo protein synthesis was also not required for the transcriptional activation of the folate opener. In addition, TRAP may not play a direct role in growth-phase-dependent regulation of transcription of the folate opener 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 opener are controlled by stringent response and do not involve TRAP.

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