The *metIC* operon involved in methionine biosynthesis in *Bacillus subtilis* is controlled by transcription antitermination

Sandrine Auger,[1] W. H. Yuen,[2] Antoine Danchin[1]†
and Isabelle Martin-Verstraete[1]

Author for correspondence: Isabelle Martin-Verstraete. Tel: +33 1 45 68 84 41. Fax: +33 1 45 68 89 48.
e-mail: iverstra@pasteur.fr

[1] Unité de Génétique des Génomes Bactériens, Institut Pasteur, URA CNRS 2171, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France

[2] Department of Chemistry, Hong Kong University, Pokfulam Road, Hong Kong

There are two major pathways for methionine biosynthesis in micro-organisms. Little is known about these pathways in *Bacillus subtilis*. The authors assigned a function to the *metI* (formerly *yjcI*) and *metC* (formerly *yjcJ*) genes of *B. subtilis* by complementing *Escherichia coli metB* and *metC* mutants, analysing the phenotype of *B. subtilis metI* and *metC* mutants, and carrying out enzyme activity assays. These genes encode polypeptides belonging to the cystathionine γ-synthase family of proteins. Interestingly, the MetI protein has both cystathionine γ-synthase and *O*-acetylhomoserine thiolyase activities, whereas the MetC protein is a cystathionine β-lyase. In *B. subtilis*, the transsulfuration and the thiolation pathways are functional *in vivo*. Due to its dual activity, the MetI protein participates in both pathways. The *metI* and *metC* genes form an operon, the expression of which is subject to sulfur-dependent regulation. When the sulfur source is sulfate or cysteine the transcription of this operon is high. Conversely, when the sulfur source is methionine its transcription is low. An S-box sequence, which is located upstream of the *metI* gene, is involved in the regulation of the *metIC* operon. Northern blot experiments demonstrated the existence of two transcripts: a small transcript corresponding to the premature transcription termination at the terminator present in the S-box and a large one corresponding to transcription of the complete *metIC* operon. When methionine levels were limiting, the amount of the full-length transcript increased. These results substantiate a model of regulation by transcription antitermination.

Keywords: sulfur metabolism, regulation by antitermination

INTRODUCTION

The molecular evolution of metabolic pathways is important for investigating the molecular aspects of the origin of life. The large number of newly sequenced genomes should allow us to increase our knowledge of metabolic biodiversity. The biosynthesis of sulfur-containing amino acids provides an example of pathways which exhibit alternative means for various organisms to synthesize their own metabolites. Indeed, cysteine and homocysteine can be synthesized directly from reduced sulfur, or by the interconversion of these two metabolites (Fig. 1). Homocysteine is then converted into methionine by a methionine synthase.

Thiolation pathways directly incorporate sulfide into *O*-acetylserine or *O*-acetylhomoserine to produce cysteine or homocysteine, respectively. These reactions are catalysed by an *O*-acetylserine thiolyase (EC 4.2.99.8) (Kredich, 1996), or by an *O*-acetylhomoserine thiolyase (EC 4.2.99.10) (Yamagata, 1989) (Fig. 1). *Saccharomyces cerevisiae* (Thomas & Surdin-Kerjan, 1997) and bacteria such as *Brevibacterium flavum* (Ozaki & Shiio, 1982) and *Leptospira meyeri* (Belfaiza *et al*., 1998) can synthesize homocysteine by thiolation. The transsulfuration pathways allow the interconversion of homocysteine and cysteine via the intermediary formation of cystathionine (Fig. 1). The synthesis of homocysteine from cysteine is the only means of transsulfuration in enteric bacteria (Greene, 1996). In *Escherichia coli* this requires the sequential action of cystathionine γ-synthase (EC 4.2.99.9), the *metB* gene product (Duchange *et al*.,
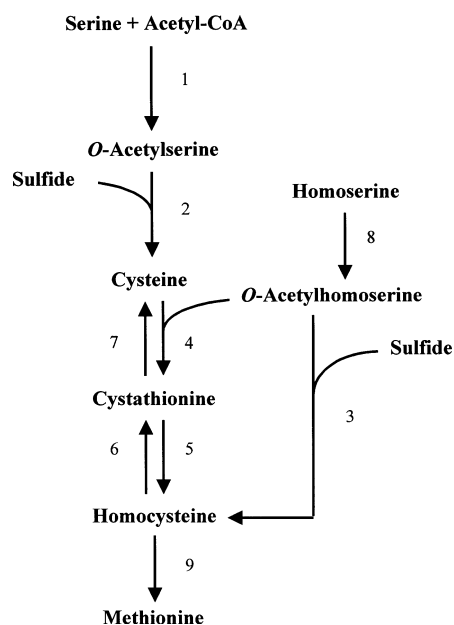
Serine + Acetyl-CoA

(Fig. 1 diagram)

Fig. 1. Biosynthetic pathways of sulfur-containing amino acids. Thiolation pathways: 2, O-acetylserine thiolyase; 3, O-acetylhomoserine thiolyase. Transsulfuration pathway: 4, cystathionine γ-synthase; 5, cystathionine β-lyase. Reverse transsulfuration pathway: 6, cystathionine β-synthase; 7, cystathionine γ-lyase. Other steps: 1, serine transacetylase; 8, homoserine transacetylase; 9, methionine synthase. Most Gram-negative bacteria use O-succinylhomoserine instead of O-acetylhomoserine.

1983), and cystathionine β-lyase (EC 4.4.1.8), the *metC* gene product (Belfaiza *et al.*, 1986). In contrast, mammalian cells only possess the homocysteine to cysteine pathway. This conversion requires a cystathionine β-synthase (EC 4.2.1.22) and a cystathionine γ-lyase (EC 4.4.1.1) (Fig. 1). *Saccharomyces cerevisiae* possesses both of the transsulfuration pathways (Thomas & Surdin-Kerjan, 1997).

A number of enzymes involved in the metabolism of cysteine, homocysteine and methionine are evolutionarily related (Alexander *et al.*, 1994). Cystathionine γ-synthase, cystathionine β-lyase, cystathionine γ-lyase, O-acetylhomoserine thiolyase and methionine γ-lyase constitute a protein family (Cherest *et al.*, 1993). The number of proteins in this family varies from organism to organism and can reach up to five or six similar polypeptides in *Bacillus anthracis*, *Bacillus halodurans*, *Clostridium acetobutylicum* and *Saccharomyces cerevisiae* (Thomas & Surdin-Kerjan, 1997). The existence of paralogues is a recurrent problem in functional genomics. Annotation procedures mostly rely on sequence comparisons. Thus, the functions of newly identified genes are thought to be identical to those of a known reference. However, this method does not address the central question of enzyme recruitment for new activities (Danchin, 1989; Jensen, 1976) and may lead to misleading assignment of functions.

Methionine plays a central role in a variety of cellular functions: it is the universal initiator of protein synthesis

and its derivative, *S*-adenosylmethionine, is involved in several cell processes including methylation (Sekowska *et al.*, 2000). Its synthesis is, therefore, tightly regulated. The enzyme producing the homoserine ester is subject to feedback inhibition by methionine and *S*-adenosylmethionine in *E. coli* and *Bacillus subtilis* (Brush & Paulus, 1971; Greene, 1996). The methionine biosynthetic pathway is also regulated at the transcriptional level. Two regulators are involved in this control in *E. coli*: the MetJ repressor and the MetR activator (Greene, 1996; Weissbach & Brot, 1991). The MetJ repressor, interacting with *S*-adenosylmethionine, binds to the Met box sequences and represses the transcription of most of the *met* genes. MetR stimulates the expression of the *metE* and *metH* genes, which encode the methionine synthases. Homocysteine markedly enhances the MetR activation of *metE* expression.

A number of *B. subtilis* genes and operons that are thought to be involved in methionine or cysteine biosynthesis contain a highly conserved sequence upstream of their coding sequence (Grundy & Henkin, 1998). This motif, the S-box, includes an element resembling an intrinsic transcriptional terminator, suggesting that regulation is controlled at the level of premature termination of transcription. Grundy & Henkin (1998) supported a model in which the 5′ portion of the leader forms an anti-antiterminator structure, which sequesters sequences required for the formation of an antiterminator, which, in turn, sequesters sequences required for the formation of the terminator, on the basis of mutational analysis of the leader region of the methionine-regulated *yitJ* gene. The anti-antiterminator is postulated to be stabilized by the binding of some unknown factor when methionine is available. This set of genes is proposed to form a new regulon, probably controlled by a global transcription termination control system (Grundy & Henkin, 1998).

We studied the involvement of the *yjcI* (now *metI*) and *yjcJ* (now *metC*) gene products in the biosynthesis of methionine in *B. subtilis*. We also demonstrated that the S-box sequence, which is located upstream of the *metI* gene, is involved in the regulation of the expression of the *metIC* operon. This motif modulates the termination of transcription in response to the availability for methionine in the medium.

METHODS

Bacterial strains and culture conditions. The *E. coli* and *B. subtilis* strains and plasmids used in this work are listed in Table 1. *E. coli* cells were grown in L-broth (LB; Sambrook *et al.*, 1989) or in minimal medium M63 (Miller, 1972) containing 1 mM L-methionine if necessary. *B. subtilis* was grown in SP medium or in minimal medium (6 mM K_2HPO_4, 4·4 mM KH_2PO_4, 0·3 mM trisodium citrate, 5 mM $MgCl_2$, 0·5% glucose, 50 mg L-tryptophan l^{-1}, 22 mg ferric ammonium citrate l^{-1}, 0·1% L-glutamine) supplemented with a sulfur source as stated: 1 mM K_2SO_4, 1 mM L-methionine, 1 mM DL-homocysteine, 0·5 mM L-cysteine or 0·1 mM L-cystathionine. Antibiotics were added at the following concentrations when

Table 1. *E. coli* and *B. subtilis* strains used in this study

Strain	Genotype*	Source†
E. coli		
WA802	*metB1 lac-3* (or *lacY1*) *galK2 galT22 supE44 hsdR* Rif^r	I. Saint-Girons‡
CAG18475	*metC162*::Tn*10 rph-1*	*E. coli* Genetic Stock Center
BL21(DE3)	F^{−} *ompT hsdS*_B (r_B[−] m_B[−]) *gal dcm* (DE3)	Novagen
B. subtilis		
168	*trpC2*	Laboratory stock
BSIP1140	*trpC2 metC′*::*lacZ cat* Δ*metC*	pDIA5509→168
BSIP1142	*trpC2 amyE*::(pA*metI′–lacZ cat*)	pDIA5510→168
BSIP1143	*trpC2 metI*::*spc*	pDIA5517→168
BSIP1153	*trpC2 amyE*::(pF*metI′–lacZ cat*)	pDIA5524→168
BSIP1154	*trpC2 amyE*::(pC*metI′–lacZ cat*)	pDIA5525→168
BSIP1162	*trpC2 amyE*::(pB*metI′–lacZ cat*)	pDIA5540→168
BSIP1163	*trpC2 amyE*::(pE*metI′–lacZ cat*)	pDIA5541→168
BSIP1164	*trpC2 metC*::*spc*	pDIA5547→168
BSIP1179	*trpC2 metI*::*spc amyE*::(pxyl *metI cat*)	pDIA5544→BSIP1143
BSIP1184	*trpC2 metI*::*spc amyE*::(pxyl *metC cat*)	pDIA5553→BSIP1164
BSIP1229	*trpC2* Δ(Sbox–*metIC*)::*aphA3*	This study
BSIP1300	*trpC2 amyE*::(pxylF*metI′–lacZ cat*)	pDIA5600→168
BSIP1301	*trpC2 metC*::*spc amyE*::(pxyl *metC cat*)	pDIA5553→BSIP1164
BSIP1302	*trpC2 amyE*::(pD*metI′–lacZ cat*)	pDIA5571→168

* *cat*, pC194 chloramphenicol acetyltransferase gene; *aphA3*, *Enterococcus faecalis* kanamycin-resistance gene; *spc*, spectinomycin-resistance gene.

† Arrows indicate construction by transformation.

‡ Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, Paris, France.

required: 100 µg ampicillin ml⁻¹; 10 µg tetracycline ml⁻¹; 5 µg chloramphenicol ml⁻¹; 100 µg spectinomycin ml⁻¹; 5 µg kanamycin ml⁻¹. Solid media were prepared by addition of 20 g Agar noble l⁻¹ (Difco). Standard procedures were used to transform *E. coli* (Sambrook *et al.*, 1989) and *B. subtilis* (Kunst & Rapoport, 1995). All experiments were performed in accordance with the European regulation requirements concerning the use of genetically modified organisms (GMO level 1 containment, agreement no. 2735).

The loss of amylase activity was detected as described by Stülke *et al*. (1997). β-Galactosidase specific activity was measured as described by Miller (1972) with cell extracts obtained by lysozyme treatment. Protein concentrations were determined by the Bradford method (Bradford, 1976). One unit of β-galactosidase activity was defined as the amount of enzyme that produces 1 nmol *o*-nitrophenol min⁻¹ at 28 °C. The mean values of at least three independent experiments are presented. Standard deviations (sᴅ) were less than 15 % of the mean.

DNA manipulations. Plasmids from *E. coli* and chromosomal DNA from *B. subtilis* were prepared according to standard procedures. Restriction enzymes, Klenow polymerase (Roche), *Taq* DNA polymerase (Roche) and phage T4 DNA ligase (Biolabs) were used as recommended by the manufacturers. DNA fragments were purified from agarose gels with the Qiaquick kit (Qiagen). DNA sequences were determined using the dideoxy chain-termination method with the Thermo Sequenase kit (Amersham).

Construction of plasmids and strains. To complement the *metB* and *metC* mutants of *E. coli*, the *B. subtilis metI* (*yjcI*) and *metC* (*yjcJ*) genes were expressed under the control of a *lac* promoter in pHT315 (Arantès & Lereclus, 1991). The coding sequences of *metI* (nucleotides +159 to +1373 relative to the transcription start point) and *metC* (nucleotides +1197 to +2543) were amplified by PCR using primers containing a 5′-*Bam*HI site or a 3′-*Eco*RI site. These fragments were inserted between the *Bam*HI and *Eco*RI sites of pHT315.

MetI (nucleotides −55 to +1730) and *metC* (nucleotides +1414 to +2975) DNA fragments were amplified by PCR using primers that introduced a *Bam*HI or *Eco*RI restriction site at the 5′- or 3′-end. These fragments were inserted into pJH101 (Ferrari *et al.*, 1983), resulting in plasmids pJH101*metI* and pJH101*metC*. A *Sma*I-restricted spectinomycin-resistance cassette (Murphy, 1985) was cloned into the *Age*I site of blunt-ended pJH101*metI* and into the *Mlu*I site of pJH101*metC*, resulting in pDIA5517 and pDIA5547, respectively. These plasmids, linearized by *Sca*I, were used to transform *B. subtilis* 168. The *metI* or the *metC* genes were disrupted by the spectinomycin resistance cassette by a double cross-over event, giving rise to strains BSIP1143 and BSIP1164, respectively (Table 1).

Plasmid pX, with its xylose-inducible promoter (Kim *et al.*, 1996), was used to complement *B. subtilis metI* and *metC* mutants with the *metI* and *metC* genes. The complete coding sequences of *metI* (nucleotides +159 to +1373) and *metC* (nucleotides +1127 to +2543) were amplified by PCR. A *Spe*I or *Bam*HI restriction site was created at the 5′- or 3′-end of the fragments. These DNA fragments were inserted into the *Spe*I and *Bam*HI sites of pX, producing pDIA5544 and pDIA5553. These plasmids were then used to transform different *B. subtilis* mutants, leading to the integration of the *metI* or *metC* gene at the *amyE* locus (Table 1).
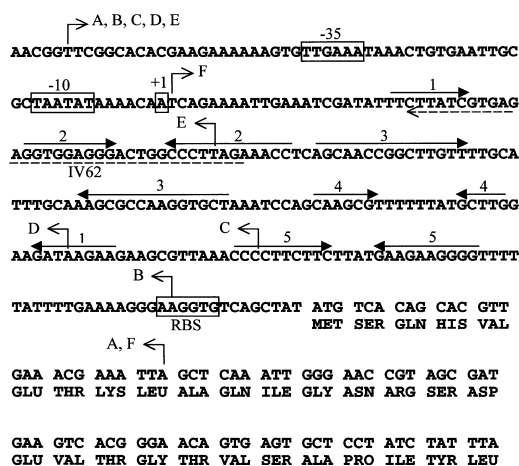
```
                    ⌐→A, B, C, D, E
                                        -35
AACGGTTCGGCACACGAAGAAAAAAGTG TTGAAA TAAACTGTGAATTGC

  -10          +1  ⌐→ F
GC TAATAT AAAACA A TCAGAAAATTGAAATCGATATTTCTTATCGTGAG
                                                      1
              E ←⌐
   2              ←       2                3
AGGTGGAGGGACTGGCCCTTAGAAACCTCAGCAACCGGCTTGTTTTGCA
    IV62
           3                4               4
TTTGCAAAGCGCCAAGGTGCTAAATCCAGCAAGCGTTTTTTATGCTTGG

 D ←⌐              C ←⌐
     1                     5
AAGATAAGAAGAAGCGTTAAACCCCTTCTTCTTATGAAGAAGGGGTTTT

        B ←⌐
TATTTTGAAAAGGG AAGGTG TCAGCTAT ATG TCA CAG CAC GTT
          RBS                   MET SER GLN HIS VAL
        A, F ⌐→
GAA ACG AAA TTA GCT CAA ATT GGG AAC CGT AGC GAT
GLU THR LYS LEU ALA GLN ILE GLY ASN ARG SER ASP


GAA GTC ACG GGA ACA GTG AGT GCT CCT ATC TAT TTA
GLU VAL THR GLY THR VAL SER ALA PRO ILE TYR LEU
```

Fig. 2. Nucleotide sequence of the *metI* upstream region. The −10 and −35 regions, the transcription initiation site (+1) and the putative *metI* ribosome-binding site (RBS) are indicated. Numbered arrows indicate the positions of inverted repeats corresponding to the helical regions homologous to S-box sequences. The dotted arrow indicates the sequence of the primer used for the primer extension experiment and broken arrows show deletion end-points of the different fusions with the *lacZ* gene.

A transcriptional *metC'–lacZ* fusion was constructed as follows: an *Eco*RI–*Bam*HI internal fragment of the *metC* gene (nucleotides +1414 to +1730), generated by PCR, was cloned into the integrative plasmid pDIA5307 (Calogero *et al.*, 1994). The resulting plasmid, pDIA5509, was integrated by a Campbell-type mechanism into the chromosome of the wild-type strain to create strain BSIP1140.

Plasmid pAC6 (Stülke *et al.*, 1997) allowed the construction of transcriptional fusions between a series of 5′ and 3′ deletions of the *metI* promoter region and the promoterless *lacZ* gene. The A (nucleotides −58 to +263), B (nucleotides −58 to +198), C (nucleotides −58 to +157), D (nucleotides −58 to +140), E (nucleotides −58 to +55) and F (nucleotides +2 to +263) regions were amplified by PCR, with the creation of *Eco*RI and *Bam*HI sites. The PCR products were inserted into pAC6 to give pDIA5510 (A), pDIA5540 (B), pDIA5525 (C), pDIA5571 (D), pDIA5541 (E) and pDIA5524 (F), respectively. These plasmids were linearized with *Sca*I, which allowed the insertion of the transcriptional *lacZ* fusions as a single copy at the *amyE* locus (Table 1). Strain BSIP1300 was constructed as follows. A DNA fragment of 3 kb corresponding to the entire transcriptional fusion pF(+2; +263)*metI'–lacZ* was amplified by PCR using pDIA5540 as a template. The *Spe*I–*Bgl*II PCR fragment obtained was inserted into the *Spe*I and *Bam*HI sites of pX (Kim *et al.*, 1996), producing pDIA5600. The transcriptional pxylF*metI'–lacZ* fusion was inserted at the *amyE* locus (Table 1).

To construct strain BSIP1229, in which the S-box and the *metIC* genes are replaced by a kanamycin-resistance cassette (Trieu-Cuot & Courvalin, 1983), a four-primer PCR procedure was used (Wach, 1996). The *aphA3* gene, encoding the kanamycin-resistance cassette, was first amplified. The regions upstream from the *metI* gene (nucleotides −1037 to −66) and downstream from the *metC* gene (nucleotides +2254 to +3246) were amplified by PCR so that 21 bp fragments were introduced at one of their ends. Three DNA fragments were

then combined: the *metI* upstream region with a 21 bp *aphA3* fragment at its 3′ end, the *metC* downstream region with a 21 bp *aphA3* fragment at its 5′ end, and the complete *aphA3* gene. The *metI* upstream region and the *metC* downstream region, overlapping the *aphA3* gene at one end, served as long primers for PCR using *aphA3* as a template. In this second PCR reaction, two external primers (5′ upstream and 3′ downstream primers) were added. The final product, corresponding to the two regions flanking the S-box–*metIC* sequence with the inserted *aphA3* cassette in between, was purified from a gel and used to transform *B. subtilis* 168 (Table 1).

RNA isolation and analysis. Total RNA was isolated from *B. subtilis* 168 grown in minimal medium supplemented with 1 mM sulfate and/or 1 mM L-methionine. Exponentially growing cells (OD_{600} 0·8) were harvested. One gram of 0·1 mm diameter glass beads (Sigma) was added. The cells were broken by shaking in a Fastprep apparatus (Bio101) for 2×30 s. Total RNA was extracted as described by Glatron & Rapoport (1972).

For the primer extension experiment, the primer IV62 (Fig. 2) labelled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP was hybridized with 10 µg RNA. Annealing was performed at 80 °C to avoid the secondary structure of RNA. The DNA primer was extended by use of reverse transcriptase and the products were analysed as described by Sambrook *et al.* (1989). The same primer was used to generate a sequence ladder (Sanger *et al.*, 1977).

For Northern blot analysis, 3 µg RNA was separated in a 1·5% denaturing agarose gel containing 2% formaldehyde, and transferred to Hybond-N+ membrane (Amersham). [α-^{32}P]ATP-labelled probes were generated with the Random Primed DNA labelling kit (Roche). Probe 1 consisted of a 197 bp fragment (nucleotides +1 to +198) covering the S-box sequence. Probe 2 corresponded to a 304 bp fragment (nucleotides +207 to +511) of the *metI* gene (Fig. 3). Northern blots were analysed and quantified with the Image Master 1D software (Amersham).

Overexpression of MetI. The *metI* gene (nucleotides +210 to +1340), amplified by PCR using primers introducing a 5′-*Nde*I and a 3′-*Xho*I site, was cloned into the pET20b⁺ vector (Novagen) which had been digested by *Nde*I and *Xho*I. The resulting plasmid, pET20b⁺*metI*, was transformed into *E. coli* WA802 (*metB1*), which carries the T7 RNA polymerase gene on pGP1-2 (Tabor & Richardson, 1985). Cells were grown at 30 °C in LB and transferred to 37 °C for 2 h to induce the expression of the *metI* gene. The pET20b⁺*metI* construct was also introduced into *E. coli* BL21(DE3) (Novagen), which contains pDIA17 (Munier *et al.*, 1991) encoding the *lacI* repressor. Transformants were grown in LB to OD_{600} 3. The expression of *metI* was induced by adding IPTG (3 mM). The transformants were incubated for a further 2 h.

Enzyme assays. Cystathionine γ-synthase catalyses the γ-replacement reaction with the acetyl ester of L-homoserine and L-cysteine. The reaction mixture contained 100 mM Tris/HCl (pH 7·6), 0·25 mM pyridoxal phosphate, 2·5 mM O-acetyl-L-homoserine, 1 mM L-cysteine and crude cellular extract, and was incubated at 30 °C. The reaction was stopped by incubating at 100 °C for 2 min. The disappearance of L-cysteine was measured by the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) method (Jocelyn, 1987). O-Acetylhomoserine thiolyase activity was assayed by measuring how much L-homocysteine was formed from O-acetyl-L-homoserine and sodium sulfide. The assay was carried out as described above,
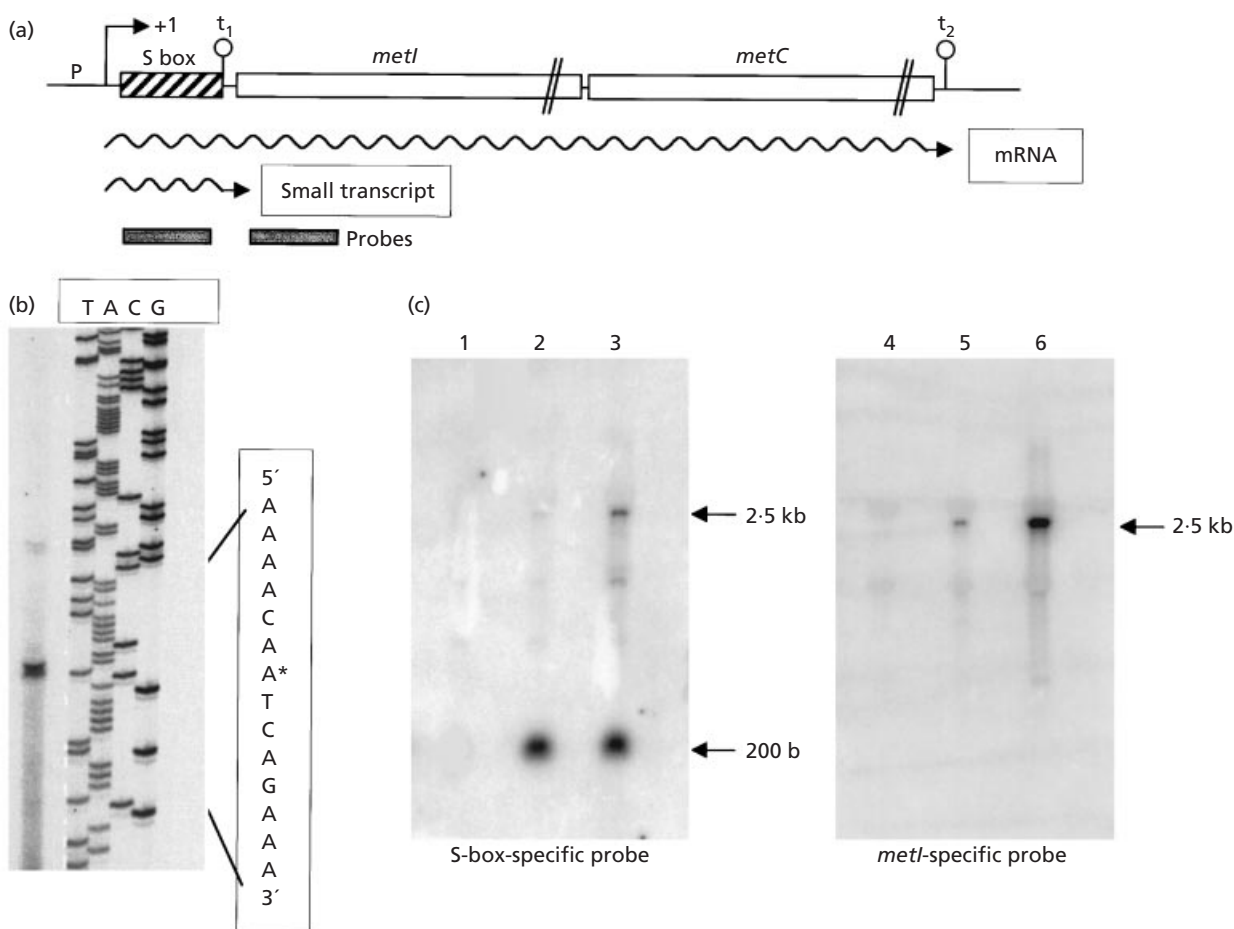
Fig. 3. Genetic organization and transcriptional regulation of the *B. subtilis metIC* operon. (a) Structure of the *metIC* operon. The promoter (P), the S-box and the transcriptional terminators (t₁ and t₂) are indicated. The two transcripts of the *metIC* operon and the DNA fragments used as probes in the Northern blot experiments are also shown. (b) Identification of the transcription start site of the *metIC* operon by primer extension. Total RNA was extracted from *B. subtilis* 168 grown in minimal medium with 1 mM sulfate as the sole sulfur source. The size of the extended product was compared to a DNA sequencing ladder of the *metIC* promoter region. The +1 site is marked by an asterisk. (c) Northern blot analysis of the *metIC* operon. *B. subtilis* 168 was grown in minimal medium with 1 mM sulfate (lanes 3 and 6) or with 1 mM sulfate and 1 mM methionine (lanes 2 and 5) as sulfur sources. The Δ(S-box–*metIC*) mutant, BSIP1229, was grown in minimal medium with 1 mM methionine (lanes 1 and 4). Lanes 1, 2 and 3 correspond to hybridization with an S-box-specific probe. Lanes 4, 5 and 6 correspond to hybridization with a *metI*-specific probe. Arrows indicate the apparent sizes of the transcripts detected.

except that cysteine was replaced with 8 mM Na₂S. The amount of L-homocysteine formed was determined by the nitroprusside reaction (Yamagata, 1987). Cystathionine β-lyase catalyses the conversion of L-cystathionine to ammonia, pyruvate and L-homocysteine. Its activity was assayed at 30 °C by measurement of the production of free thiol groups with DTNB (Uren, 1987). The reaction mixture contained 100 mM Tris/HCl (pH 9·0), 0·2 mM DTNB, 0·2 mM pyridoxal phosphate, 2 mM substrate and crude cellular extract.

RESULTS

YjcI and YjcJ polypeptides belong to a pyridoxal-dependent enzyme family

Cystathionine γ-synthase, cystathionine β-lyase, cystathionine γ-lyase and O-acetylhomoserine thiolyase be-long to the same protein family (Cherest *et al.*, 1993). These enzymes, of about 400 aa, use the same cofactor, pyridoxal phosphate, which is attached to a lysine residue (Martel *et al.*, 1987). The sequence surrounding this residue is highly conserved and can be used as a signature pattern to detect this class of enzymes: [DQ]-[LIVMF]-X₃-[STAGC]-[STAGCI]-T-K-[FYWQ]-[LIVMF]-X-G-[HQ]-[SGNH] (http://www.expasy.ch/prosite). Analysis of the complete *B. subtilis* genome sequence revealed the presence of three genes, *yjcI*, *yjcJ* and *yrhB*, encoding proteins with similar signature patterns. YjcI, YjcJ and YrhB are proteins of 373, 390 and 379 aa, respectively. A multiple alignment of these polypeptides with the *E. coli* cystathionine γ-synthase (MetB) and cystathionine β-lyase (MetC) enzymes showed that the *yjcI*, *yjcJ* and *yrhB* gene products share

Table 2. Complementation of *E. coli metB* and *metC* mutants by *B. subtilis yjcI* and *yjcJ* genes

Precultures were grown in M63 medium with 1 mM sulfate plus 1 mM methionine. Cells were centrifuged and resuspended at OD_{600} 0·1 in M63 medium with 1 mM sulfate or 1 mM sulfate plus 1 mM methionine as sulfur source. OD_{600} was measured after 12 h growth at 37 °C.

E. coli strain (relevant genotype)	Plasmid	OD_{600} after 12 h	
		Sulfate	Sulfate + Methionine
WA802 (*metB1*)	pHT315	0·12	4·0
	pHT315 *yjcI*	1·00	4·0
	pHT315 *yjcJ*	0·14	4·5
CAG18475 (*metC162*::Tn*10*)	pHT315	0·20	1·8
	pHT315 *yjcI*	0·09	2·0
	pHT315 *yjcJ*	2·20	2·2

Table 3. Phenotype of the *B. subtilis yjcI* and *yjcJ* mutants

Cells were grown in minimal medium containing the various sulfur sources indicated. For expression of the genes under the control of the *xylA* promoter, minimal medium contained fructose instead of glucose and 0·1% xylose. NG, No growth; ND, not determined.

Strain	Relevant genotype	Doubling time (min)				
		Sulfate (1 mM)	Cysteine (0·5 mM)	Cystathionine (0·1 mM)	Homocysteine (1 mM)	Methionine (1 mM)
168		48	57	50	53	55
BSIP1143	*yjcI*::*spc*	NG	NG	NG	63	55
BSIP1179	*yjcI*::*spc amyE*::pxyly*jcI*	56	ND	NG	ND	ND
BSIP1184	*yjcI*::*spc amyE*::pxyly*jcJ*	NG	ND	105	ND	ND
BSIP1164	*yjcJ*::*spc*	55	57	NG	63	54
BSIP1301	*yjcJ*::*spc amyE*::pxyly*jcJ*	58	ND	60	ND	ND

38–42% identity with MetB, and 27–30% identity with MetC. The *yjcI* and *yjcJ* genes are adjacent on the chromosome. The nucleotide sequence of *yjcI* overlaps that of *yjcJ* at the sequence TTGA, where TTG is the initiation codon of *yjcJ* and TGA is the stop codon of *yjcI*. This strongly suggests that these two genes form an operon.

Complementation of *E. coli metB* and *metC* mutants with *B. subtilis yjcI* and *yjcJ* genes

E. coli metB and *metC* mutants lacking cystathionine *γ*-synthase or cystathionine *β*-lyase activity, respectively, are methionine auxotrophs (Greene, 1996). To determine the function of the *B. subtilis yjcI* and *yjcJ* genes, we first tried to complement *E. coli metB* and *metC* mutants. The *yjcI* and *yjcJ* genes were cloned into the replicative vector pHT315, under the control of the *lac* promoter. The plasmids pHT315, pHT315*yjcI* and pHT315*yjcJ* were introduced into the *E. coli* mutants WA802 (*metB1*) and CAG18475 (*metC162*::Tn*10*) (Table 1). In a minimal medium with sulfate as sulfur source, pHT315*yjcI* restored the growth of the *metB* mutant, whereas pHT315*yjcJ* allowed the growth of the *metC* mutant (Table 2). pHT315 alone did not complement these mutants. In a methionine-supplemented medium, all strains were able to grow. These results strongly suggest that the YjcI product is a cystathionine *γ*-synthase and that the YjcJ product is a cystathionine *β*-lyase.

Phenotype of the *yjcI* and *yjcJ B. subtilis* mutant strains

To investigate the physiological role of the *yjcI* and *yjcJ* genes in homocysteine biosynthesis, we disrupted their coding region by double-crossover events that resulted in marker replacement. Growth of the wild-type, the BSIP1143 (*yjcI*::*spc*) and the BSIP1164 (*yjcJ*::*spc*) strains was compared in minimal medium containing various sulfur sources (Table 3). The *yjcI* mutant was unable to grow in the presence of sulfate, cysteine or cystathionine as sole sulfur source, whereas its growth in the presence of homocysteine or methionine was similar to that of the wild-type strain. In contrast, the mutant lacking *yjcJ* grew as well as the wild-type in the presence of sulfate, cysteine, homo-

cysteine or methionine as sole sulfur source. However, this mutant did not grow in the presence of cystathionine.

The absence of growth of the *yjcI* mutant in the presence of cystathionine may be due to a polar effect on the *yjcJ* gene, which is located downstream of *yjcI* on the *B. subtilis* chromosome. To test this hypothesis, the *yjcI* or the *yjcJ* genes, expressed under the control of the *xylA* promoter, were integrated as a single copy at the *amyE* locus of the BSIP1143 (*yjcI*::*spc*) and BSIP1164 (*yjcJ*::*spc*) mutants (Table 1). The introduction of the *yjcJ* gene *in trans* restored the growth of both the *yjcJ* and *yjcI* mutants in the presence of cystathionine. The *yjcI* gene *in trans* allowed the *yjcI* mutant to grow in the presence of sulfate, but not in the presence of cystathionine (Table 3). Thus, the lack of growth of the *yjcI* mutant with cystathionine was a consequence of a polar effect on the expression of the *yjcJ* gene.

The *E. coli metB* mutant and the *B. subtilis yjcI* mutant are not able to grow on sulfate or cysteine. Unexpectedly, the *B. subtilis yjcJ* mutant was not auxotrophic for methionine, in contrast to the *E. coli metC* mutant. The fact that the YjcJ protein was not essential for homocysteine biosynthesis when sulfate was the sole sulfur source indicated that some other pathway must ensure the conversion of *O*-acetylhomoserine to homocysteine in *B. subtilis*. We hypothesized that the product of the *yrhB* gene, which shares 52 % identity with YjcJ, was involved in this pathway. To test this possibility, a double *yjcJ yrhB* mutant was constructed. In media containing sulfate, this strain showed the same growth rate as the *yjcJ* mutant, indicating that the YrhB polypeptide was not necessary for homocysteine biosynthesis (data not shown). Another hypothesis was that the YjcI polypeptide has both cystathionine γ-synthase and *O*-acetylhomoserine thiolyase activities. Therefore, the enzyme activity of the YjcI and YjcJ polypeptides was tested.

YjcJ, an enzyme with cystathionine β-lyase activity, and YjcI, an enzyme with both cystathionine γ-synthase and *O*-acetylhomoserine thiolyase activities

To avoid cystathionine β-lyase activity due to MetC, *E. coli* CAG18475(*metC162*::Tn*10*) was used to overproduce the YjcJ polypeptide. Cystathionine β-lyase activity, using cystathionine as substrate, was observed in crude extracts of cells transformed with pHT315*yjcJ* [16·2 µmol free thiol groups min^{-1} (mg protein)$^{-1}$]. Enzyme activity was also observed with DL-djenkolic acid [18·8 µmol free thiol groups min^{-1} (mg protein)$^{-1}$], cystine [4·2 µmol free thiol groups min^{-1} (mg protein)$^{-1}$] and lanthionine (0·8 µmol free thiol groups min^{-1} (mg protein)$^{-1}$]. The activities detected in crude extracts of the same strain transformed with pHT315 were lower than 0·1 µmol free thiol groups min^{-1} (mg protein)$^{-1}$. These results permitted us to conclude that *yjcJ* encodes a cystathionine β-lyase. We propose to rename this gene *metC*. Consequently, the former *metC* gene, encoding the probable methionine synthase, was renamed *metE*

according to *SubtiList* (http://genolist.pasteur.fr/SubtiList).

Cystathionine γ-synthase activity of the YjcI polypeptide was assayed in an *E. coli metB* mutant (WA802). *E. coli* cells transformed with pET20b^{+}*yjcI* exhibited cystathionine γ-synthase activity with *O*-acetylhomoserine as substrate [1·5 µmol cysteine min^{-1} (mg protein)$^{-1}$]. Under the same conditions, no detectable activity [≤10^{-3} µmol cysteine min^{-1} (mg protein)$^{-1}$] was obtained using *O*-succinylhomoserine, or in crude extracts of cells harbouring pET20b^{+}. *O*-Acetylhomoserine thiolyase activity of YjcI was tested in *E. coli* strain BL21(DE3). Crude extracts of BL21(DE3) transformed with pET20b^{+}*yjcI* showed *O*-acetylhomoserine thiolyase [7 µmol homocysteine min^{-1} (mg protein)$^{-1}$] and *O*-succinylhomoserine thiolyase [6·7 µmol homocysteine min^{-1} (mg protein)$^{-1}$] activities. In crude extracts of the same strain transformed with pET20b^{+} vector alone, the activities were lower than 0·1 µmol homocysteine min^{-1} (mg protein)$^{-1}$. The YjcI polypeptide has both cystathionine γ-synthase and *O*-acetylhomoserine thiolyase activities *in vitro*.

To investigate whether the YjcI polypeptide can provide *O*-acetylhomoserine thiolyase activity *in vivo*, we tested the ability of the YjcI polypeptide to complement a *B. subtilis* Δ*yjcIJ* mutant (BSIP1229) by introducing pHT315*yjcI* into this strain. Growth experiments in liquid minimal medium showed that the *yjcI* gene restored the growth of the Δ*yjcIJ* mutant in the presence of sulfate as sole sulfur source. The doubling time (50 min) was similar to that of the wild-type strain in the same conditions. Thus, the YjcI enzyme also has *O*-acetylhomoserine thiolyase activity *in vivo* which allows it to bypass the intermediate cystathionine to form homocysteine (Fig. 1). The *B. subtilis* YjcI and the *E. coli* MetB polypeptides have different functions. We therefore propose to rename *yjcI metI* rather than *metB*.

Regulation of the expression of the *metIC* operon

To study the regulation of the *metI* and *metC* genes in response to sulfur availability, *metI*′–*lacZ* and *metC*′–*lacZ* transcriptional fusions were constructed. The *metI*′–*lacZ* fusion was inserted as a single copy at the *amyE* locus of *B. subtilis* 168 (strain BSIP1142), and the *metC*′–*lacZ* fusion was integrated at the homologous *metC* locus by a Campbell-type mechanism (strain BSIP1140), leading to the disruption of this gene. The level of β-galactosidase activity was high in the presence of sulfate but 11- to 13-fold lower when both sulfate and methionine were present in the medium (Table 4). The presence of cysteine, cystathionine or homocysteine as sulfur sources led to a high level of expression of the two fusions. The regulation of expression of the *metI* and *metC* genes occurs at the transcriptional level. The decrease of expression of these genes in the presence of methionine substantiates the role of the MetI and MetC polypeptides in the methionine biosynthetic pathway.

The *metI* and *metC* genes, which are adjacent in the *B. subtilis* chromosome, are co-regulated (Table 4). To

Table 4. Expression of transcriptional pA*metI'–lacZ* and *metC'–lacZ* fusions in the presence of different sulfur sources

Cells were grown in minimal medium containing the various sulfur sources indicated. Values are the means from at least three independent experiments. NG, No growth.

Sulfur source	β-Galactosidase activity [U (mg protein)$^{-1}$]	
	BSIP1142 *amyE*::pA*metI'–lacZ*	BSIP1140 *metC'–lacZ*
Sulfate (1 mM)	515	265
Methionine (1 mM)	100	80
Sulfate (1 mM) + Methionine (1 mM)	45	20
Cystathionine (0·1 mM)	430	NG
Cysteine (0·5 mM)	475	245
Homocysteine (1 mM)	340	330

Table 5. Effect of sulfate and methionine on the expression of different *metI'–lacZ* transcriptional fusions

Cells were grown in minimal medium containing the indicated sulfur sources at 1 mM. Samples were taken during the exponential growth phase at $OD_{600} \sim 1$. Values are the means from at least three independent experiments.

Strain	Fusion at the *amyE* locus*	β-Galactosidase activity [U (mg protein)$^{-1}$]			Regulation factor†
		Sulfate	Methionine	Sulfate + Methionine	
BSIP1142	pA(−58; +263) *metI'–lacZ*	515	100	45	11
BSIP1162	pB(−58; +198) *metI'–lacZ*	85	8	4	21
BSIP1154	pC(−58; +157) *metI'–lacZ*	520	530	560	0·9
BSIP1302	pD(−58; +140) *metI'–lacZ*	470	450	550	0·8
BSIP1163	pE(−58; +55) *metI'–lacZ*	390	390	355	1·1
BSIP1153	pF(+2; +263) *metI'–lacZ*	0·5	2	0·5	1

* The DNA fragment of the *metI* promoter region is indicated and the nucleotides are numbered taking the transcriptional start site as +1.

† β-Galactosidase activity ratio sulfate:sulfate + methionine.

confirm the existence of a *metIC* operon, we carried out Northern blot experiments with total RNA isolated from *B. subtilis* 168 grown with sulfate as sole sulfur source. Hybridization with a *metI*-specific probe revealed a single 2·5 kb transcript, which corresponds to the size of a bicistronic *metIC* mRNA (Fig. 3c).

Identification of the *metI* promoter

The DNA sequence of the *metI* promoter region is presented in Fig. 2. The translation initiation codon is an ATG preceded by a reasonable ribosome-binding site. The S-box motif is located upstream of the *metI* translation start site. The 5′-end of the transcript was identified by primer extension using an oligonucleotide complementary to a region near the 5′-end of the S-box (Fig. 2) and total RNA from *B. subtilis* 168 grown in minimal medium with sulfate as sulfur source. Transcription is initiated at an A residue located 211 bp upstream of the translational start site (Fig. 3b). The deduced −35 (TTGAAA) and −10 (TAATAT) regions

of the promoter, boxed in Fig. 2, are similar to the consensus sequence of σ^A-dependent promoters.

To confirm the position of the promoter, a transcriptional fusion was constructed between the *lacZ* gene and the *metI* promoter F region (nucleotides +2 to +263 relative to the transcription start site) with deletion of the −35 and −10 regions. The fusion was integrated at the *amyE* locus of *B. subtilis*. Compared to the pA(−58; +263)*metI'–lacZ* fusion, this fusion gave very low β-galactosidase activities in minimal media in the presence of either methionine or sulfate as sulfur source (Table 5). Therefore, deletion of the mapped promoter abolished transcription of the *metI'–lacZ* fusion, indicating the existence of a single promoter.

Role of the S-box in the regulation of the expression of the *metIC* operon

The *cis*-acting elements required for the regulation of the *metIC* operon are present in the pA(−58; +263)*metI'–lacZ* transcriptional fusion. This region

contains the *metIC* promoter and the S-box sequence. To determine the minimal sequence necessary for full regulation of the operon, *metI* promoter regions containing various 3′ deletions were fused with the promoterless *lacZ* gene. The fusion end-points are indicated in Fig. 2. These fusions were introduced as a single copy at the *amyE* locus of *B. subtilis* 168.

Expression of the pB(−58; +198)*metI′–lacZ* fusion, which contains the entire S-box sequence, was 21-fold higher in the presence of sulfate than in the presence of sulfate plus methionine (Table 5). In contrast, pC(−58; +157)*metI′–lacZ* and pD(−58; +140)*metI′–lacZ* deletions caused a high *β*-galactosidase activity during growth in the presence or absence of methionine. These results indicate that the DNA fragment located between nucleotides +157 and +198 is necessary for the regulation in response to methionine availability. This region contains a putative factor-independent terminator (helix 5, Fig. 2) probably involved in premature transcription termination (Grundy & Henkin, 1998). The pE(−58; +55)*metI′–lacZ* fusion only contains the promoter and the 5′ end of the S-box (half of helix 1 and half of helix 2). This fusion also exhibited high-level constitutive expression, indicating that the *metIC* promoter was constitutively active.

To confirm the respective role of the promoter and the S-box in the regulation of the *metIC* operon, we constructed a transcriptional fusion in which the *metI* promoter was replaced by the *xylA* promoter. The resulting construct was integrated at the *amyE* locus, giving strain BSIP1300. The *β*-galactosidase activity was measured after growth in minimal medium with 0·1% xylose. The level of *β*-galactosidase activity was 75 U (mg protein)$^{-1}$ in the presence of sulfate, 28 U (mg protein)$^{-1}$ in the presence of methionine, and 10 U (mg protein)$^{-1}$ in the presence of sulfate and methionine. Although the level of expression was lower than that observed for the native pA*metI′–lacZ* fusion, the trend was similar. The S-box sequence is, therefore, sufficient to mediate the regulation of the *metIC* operon expression.

Expression of the *metIC* operon is controlled by transcription antitermination

In transcription antitermination systems, a transcriptional terminator forms in the leader region of the mRNA and determines whether transcription will terminate or continue. Northern blot experiments were performed to investigate the existence of a long *metIC* transcript and a short transcript. Total RNA was isolated from a culture of *B. subtilis* 168 grown in the presence of sulfate, or in the presence of sulfate plus methionine. Probes for the S-box and *metI* were used to detect specific *metIC* transcripts (Fig. 3). When sulfate was the sole sulfur source, a 2·5 kb transcript was detected by both probes (Fig. 3c, lanes 3 and 6). This transcript was not detected in RNA extracted from the Δ(S-box–*metIC*) mutant, BSIP1229 (Fig. 3c, lanes 1 and 4). It corresponds in length to a transcript initiated at the

metI promoter and terminated at the terminator located downstream of *metC* (Fig. 3a). In the presence of sulfate plus methionine, the amount of the 2·5 kb transcript decreased tenfold with the *metI* probe and eightfold with the S-box probe (Fig. 3c, lanes 2 and 5). These results are consistent with the regulation of the *metIC* operon in response to methionine availability observed with *lacZ* transcriptional fusions. In addition to this full length mRNA, a 200 bp transcript was specifically detected by the S-box probe. Its presence was detectable with the same intensity regardless of whether methionine was present or not (Fig. 3c, lanes 2 and 3). This transcript was not detected by the *metI*-specific probe or with RNA extracted from BSIP1229 [Δ(S-box–*metIC*)] (Fig. 3c, lanes 1, 4, 5 and 6). A transcript of this size would be expected if RNA synthesis was initiated from the *metI* promoter and stopped at the terminator (helix 5) in the 3′-end of the S-box. The presence of the long and short transcripts correlates with a model of transcription antitermination.

DISCUSSION

The complete genome sequence of *B. subtilis* revealed three genes, *yjcI*, *yjcJ* and *yrhB*, encoding putative pyridoxal-dependent enzymes belonging to the cystathionine *γ*-synthase family (Cherest *et al.*, 1993). We have shown that the YjcI (MetI) and YjcJ (MetC) proteins have central roles in methionine biosynthesis. In *B. subtilis*, homocysteine is synthesized by two metabolic pathways. The first one corresponds to the transsulfuration pathway present in enterobacteria, except that *O*-acetylhomoserine is used as the substrate instead of *O*-succinylhomoserine (Kanzaki *et al.*, 1986). Enzyme assays revealed that the MetI protein has cystathionine *γ*-synthase activity and could catalyse the synthesis of cystathionine from *O*-acetylhomoserine and cysteine (Fig. 1). The complementation of the *E. coli metB* mutant by the *B. subtilis metI* gene was probably due to marginal use of the *E. coli* substrate *O*-succinylhomoserine. The MetC protein has cystathionine *β*-lyase activity and catalyses the biosynthesis of homocysteine from cystathionine. Cystathionine appears to be an intermediate metabolite in the methionine biosynthesis pathway of *B. subtilis*. Disruption of the *metC* gene prevented *B. subtilis* from growing on cystathionine, as expected for a mutant inactivated in cystathionine *β*-lyase. Interestingly, the MetC protein is not essential for homocysteine synthesis in the presence of sulfate (Table 3), indicating that another pathway exists. YrhB, a MetI/MetC-like protein, which could be implicated in methionine degradation, is not involved.

The phenotype of the *metI* mutant (Table 3) shows that the MetI protein is important for the second pathway. The MetI polypeptide also exhibited *O*-acetylhomoserine thiolyase activity. This enzyme catalyses the synthesis of homocysteine from sulfide and *O*-acetylhomoserine. Complementation with the *metI* gene allowed the *B. subtilis* Δ*metIC* mutant to grow with sulfate as its sole sulfur source. Therefore, the thiolation

pathway (Fig. 1), which bypasses the formation of cystathionine, is functional *in vivo* in *B. subtilis*. The MetI protein has a dual cystathionine γ-synthase/O-acetylhomoserine thiolyase activity. O-Acetylhomoserine thiolyases from micro-organisms such as *Corynebacterium acetophilum* (Murooka *et al.*, 1977), *Leptospira meyeri* (Belfaiza *et al.*, 1998), *Neurospora crassa* (Kerr, 1971), *Saccharomyces cerevisiae* (Yamagata & Takeshima, 1976) and *Schizosaccharomyces pombe* (Yamagata, 1984) do not catalyse the synthesis of cystathionine. It will be interesting to study the enzymic properties of MetI-like proteins in bacilli and other Gram-positive bacteria, since the purified *Bacillus sphaericus* cystathionine γ-synthase has O-acetylhomoserine thiolyase activity *in vitro* (Kanzaki *et al.*, 1987). In *Pseudomonas aeruginosa* and yeast, the thiolation pathway appears to be the major route for homocysteine synthesis, although the transsulfuration pathway also exists which implicates other genes. Whether the O-acetylhomoserine thiolyase activity of MetI represents a major or an alternative pathway in *B. subtilis* remains to be clarified. In particular, the possible existence of regulatory mechanisms modulating the MetI activity deserves further investigations.

The MetI protein shares more similarities with the cystathionine γ-synthases of *E. coli* (41·8 % identity) and *Corynebacterium glutamicum* (41·1 % similarity) than with the O-acetylhomoserine thiolyases of *Leptospira meyeri* (28 % similarity) and *Saccharomyces cerevisiae* (29·7 % similarity). In addition, O-acetylhomoserine thiolyases contain a 30 aa insertion, in their central region, which is not present in the MetI sequence (Cherest *et al.*, 1993). The hypothesis of Jensen (1976), concerning the molecular evolution of metabolic pathways, suggested that primitive enzymes have a very broad specificity and have further evolved to become more specific and efficient catalysts. While the MetI protein is more closely related to cystathionine γ-synthases, its binding site for thiol compounds is poorly specific because either cysteine or sulfide can be used as a sulfur donor. We propose that this protein has retained some of the ancestral properties of a primitive homocysteine synthase, the common ancestor of cystathionine γ-synthases and O-acetylhomoserine thiolyases (Cherest *et al.*, 1993).

In *E. coli*, the *metC* gene corresponds to a single transcriptional unit and the *metB* gene forms an operon with *metL*, which encodes a bifunctional aspartokinase–homoserine dehydrogenase (Greene, 1996). In *B. subtilis*, the *metI* and *metC* genes, which probably correspond to the historically named *metA* locus (Anagnostopoulos *et al.*, 1993), are organized in an operon. A search of other sequences in the databases revealed a similar arrangement in *Staphylococcus aureus*, *Clostridium acetobutylicum*, *Bacillus halodurans* and *Bacillus anthracis*, indicating that this organization is conserved in a number of Gram-positive bacteria. An S-box motif is located upstream of the *metIC*-like operons in all of these organisms, except in *Staphylococcus aureus*.

Genes encoding amino acid biosynthesis enzymes in bacteria are generally expressed when the levels of the corresponding amino acid decrease. The *metIC* operon responds specifically to methionine availability and not to cysteine. Surprisingly, the expression of this operon is high in the presence of homocysteine, the methionine precursor (Fig. 1). Little is known about the regulation of expression of the other steps of the methionine biosynthetic pathway with the exception of the *metK* gene, which is two- to threefold regulated in response to exogenous methionine (Yocum *et al.*, 1996). Further work is needed to estimate the metabolic fluxes and to study in more detail the different pathways connected to methionine biosynthesis.

The S-box has a key role in the control of transcription of the *metIC* operon, encoding two major steps of methionine biosynthesis. This motif is also found upstream of the genes encoding methionine synthase (*metE*) and S-adenosylmethionine synthase (*metK*), and is important for the regulation of the *yitJ* gene, which is thought to be involved in the synthesis of methionine (Grundy & Henkin, 1998). In contrast, the regulation of expression of the *cysH* operon, which encodes the first steps of the cysteine biosynthesis, is independent of the S-box sequence located upstream of this operon (Mansilla *et al.*, 2000). The S-box is not found in the upstream region of the other genes involved in cysteine biosynthesis. Therefore, the S-box system is probably devoted to the control of methionine metabolism, rather than to that of sulfur availability.

Analysis of the *metIC* operon revealed that its induction is independent of the promoter located upstream of the S-box sequence, but is dependent on the terminator of the leader region (Fig. 3, Table 5). We used Northern blot analysis to demonstrate that two transcripts exist: a small transcript corresponding to termination of transcription at the end of the S-box motif and a large one corresponding to transcription of the complete *metIC* operon. Therefore, the intrinsic terminator (helix 5) of the S-box motif is functional in *B. subtilis*. When methionine is limiting, the amount of full-length *metIC* transcript increases (Fig. 3c). This indicates that *metIC* expression is induced by partial alleviation of transcriptional termination at the S-box terminator in response to methionine availability. In contrast, the amount of the small transcript appears to be the same in the presence or absence of methionine. The secondary structure of the leader region could stabilize the small RNA. It is common for gene expression to be controlled at the level of transcription termination in prokaryotes (Henkin, 1996). In *B. subtilis*, the *bgl–sac* type of catabolic operons, the *hut* operon, the *pyr* and *trp* biosynthetic operons, the *ssu* (sulfonate–sulfur utilization) operon and the T-box controlled genes are all regulated by systems involving alternative RNA structures (Henkin, 1996; Oda *et al.*, 2000; Rutberg, 1997; van der Ploeg *et al.*, 2001). The critical differences between these systems are the molecules interacting with the leader region (tRNA or RNA-binding proteins) and how they modulate terminator and antiterminator

formation. Further analyses are required to identify the regulatory factor(s) and the nature of the effector molecule involved in the control of the S-box regulon in response to methionine availability.

REFERENCES

Alexander, F. W., Sandmeier, E., Mehta, P. K. & Christen, P. (1994). Evolutionary relationships among pyridoxal-5′-phosphate-dependent enzymes. Regio-specific alpha, beta and gamma families. *Eur J Biochem* **219**, 953–960.

Anagnostopoulos, C., Piggot, P. J. & Hoch, J. A. (1993). The genetic map of *Bacillus subtilis*. In *Bacillus subtilis and Other Gram-positive Bacteria*, pp. 425–461. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Mircobiology.

Arantès, O. & Lereclus, D. (1991). Construction of cloning vectors for *Bacillus thuringiensis*. *Gene* **108**, 115–119.

Belfaiza, J., Parsot, C., Martel, A., de la Tour, C. B., Margarita, D., Cohen, G. N. & Saint-Girons, I. (1986). Evolution in biosynthetic pathways: two enzymes catalyzing consecutive steps in methionine biosynthesis originate from a common ancestor and possess a similar regulatory region. *Proc Natl Acad Sci U S A* **83**, 867–871.

Belfaiza, J., Martel, A., Margarita, D. & Saint-Girons, I. (1998). Direct thiolation for methionine biosynthesis in *Leptospira meyeri*. *J Bacteriol* **180**, 250–255.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.

Brush, A. & Paulus, H. (1971). The enzymic formation of O-acetylhomoserine in *Bacillus subtilis* and its regulation by methionine and *S*-adenosylmethionine. *Biochem Biophys Res Commun* **45**, 735–741.

Calogero, S., Gardan, R., Glaser, P., Schweitzer, J., Rapoport, G. & Débarbouillé, M. (1994). RocR, a novel regulatory protein controlling arginine utilization in *Bacillus subtilis*, belongs to the NtrC/NifA family of transcriptional activators. *J Bacteriol* **176**, 1234–1241.

Cherest, H., Thomas, D. & Surdin-Kerjan, Y. (1993). Cysteine biosynthesis in *Saccharomyces cerevisiae* occurs through the transsulfuration pathway which has been built up by enzyme recruitment. *J Bacteriol* **175**, 5366–5374.

Danchin, A. (1989). Homeotopic transformation and the origin of translation. *Prog Biophys Mol Biol* **54**, 81–86.

Duchange, N., Zakin, M. M., Ferrara, P., Saint-Girons, I., Park, I., Tran, S. V., Py, M. C. & Cohen, G. N. (1983). Structure of the *metJBLF* cluster in *Escherichia coli* K12. Sequence of the *metB* structural gene and of the 5′- and 3′-flanking regions of the *metBL* operon. *J Biol Chem* **258**, 14868–14871.

Ferrari, F. A., Nguyen, A., Lang, D. & Hoch, J. A. (1983).

Construction and properties of an integrable plasmid for *Bacillus subtilis*. *J Bacteriol* **154**, 1513–1515.

Glatron, M.-F. & Rapoport, G. (1972). Biosynthesis of the parasporal inclusion of *Bacillus thuringiensis*: half-life of its corresponding messenger RNA. *Biochimie* **54**, 1291–1301.

Greene, R. C. (1996). Biosynthesis of methionine. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 542–560. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.

Grundy, F. J. & Henkin, T. M. (1998). The S box regulon: a new global transcription termination control system for methionine and cysteine biosynthesis genes in Gram-positive bacteria. *Mol Microbiol* **30**, 737–749.

Henkin, T. M. (1996). Control of transcription termination in prokaryotes. *Annu Rev Genet* **30**, 35–57.

Jensen, R. A. (1976). Enzyme recruitment in evolution of new function. *Annu Rev Microbiol* **30**, 409–425.

Jocelyn, P. C. (1987). Spectrophotometric assay of thiols. *Methods Enzymol* **143**, 44–67.

Kanzaki, H., Kobayashi, M., Nagasawa, T. & Yamada, H. (1986). Distribution of two kinds of cystathionine γ-synthase in various bacteria. *FEMS Microbiol Lett* **33**, 65–68.

Kanzaki, H., Kobayashi, M., Nagasawa, T. & Yamada, H. (1987). Purification and characterization of cystathionine γ-synthase type II from *Bacillus sphaericus*. *Eur J Biochem* **163**, 105–112.

Kerr, D. S. (1971). O-Acetylhomoserine thiolase from *Neurospora*. Purification and consideration of its function in homocysteine and methionine synthesis. *J Biol Chem* **246**, 95–102.

Kim, L., Mogk, A. & Schumann, W. (1996). A xylose-inducible *Bacillus subtilis* integration vector and its application. *Gene* **181**, 71–76.

Kredich, N. M. (1996). Biosynthesis of cysteine. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 514–527. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.

Kunst, F. & Rapoport, G. (1995). Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *J Bacteriol* **177**, 2403–2407.

Mansilla, M. C., Albanesi, D. & de Mendoza, D. (2000). Transcriptional control of the sulfur-regulated *cysH* operon, containing genes involved in L-cysteine biosynthesis in *Bacillus subtilis*. *J Bacteriol* **182**, 5885–5892.

Martel, A., Bouthier de la Tour, C. & Le Goffic, F. (1987). Pyridoxal 5′-phosphate binding site of *Escherichia coli* β-cystathionase and cystathionine γ-synthase: comparison of their sequences. *Biochem Biophys Res Commun* **147**, 565–571.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Munier, H., Gilles, A. M., Glaser, P., Krin, E., Danchin, A., Sarfati, R. & Barzu, O. (1991). Isolation and characterization of catalytic and calmodulin-binding domains of *Bordetella pertussis* adenylate cyclase. *Eur J Biochem* **196**, 469–474.

Murooka, Y., Kakihara, K., Miwa, T., Seto, K. & Harada, T. (1977). O-Alkylhomoserine synthesis catalyzed by O-acetylhomoserine thiolase in microorganisms. *J Bacteriol* **130**, 62–73.

Murphy, E. (1985). Nucleotide sequence of a spectinomycin adenyltransferase AAD(9) determinant from *Staphylococcus aureus* and its relationship to AAD(3″) (9). *Mol Gen Genet* **200**, 33–39.

Oda, M., Kobayashi, N., Ito, A., Kurusu, Y. & Taira, K. (2000). *cis*-Acting regulatory sequences for antitermination in the transcript

of the *Bacillus subtilis hut* operon and histidine-dependent binding of HutP to the transcript containing the regulatory sequences. *Mol Microbiol* **35**, 1244–1254.

Ozaki, H. & Shiio, I. (1982). Methionine biosynthesis in *Brevibacterium flavum*: properties and essential role of *O*-acetylhomoserine thiolase. *J Biochem* **91**, 1163–1171.

Rutberg, B. (1997). Antitermination of transcription of catabolic operons. *Mol Microbiol* **23**, 413–421.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463–5467.

Sekowska, A., Kung, H. F. & Danchin, A. (2000). Sulfur metabolism in *Escherichia coli* and related bacteria: facts and fiction. *J Mol Microbiol Biotechnol* **2**, 145–177.

Stülke, J., Martin-Verstraete, I., Zagorec, M., Rose, M., Klier, A. & Rapoport, G. (1997). Induction of the *Bacillus subtilis ptsGHI* operon by glucose is controlled by a novel antiterminator, GlcT. *Mol Microbiol* **25**, 65–78.

Tabor, S. & Richardson, C. C. (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci U S A* **82**, 1074–1078.

Thomas, D. & Surdin-Kerjan, Y. (1997). Metabolism of sulfur amino acids in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **61**, 503–532.

Trieu-Cuot, P. & Courvalin, P. (1983). Nucleotide sequence of the *Streptococcus faecalis* plasmid gene encoding the 3′5″-aminoglycoside phosphotransferase type III. *Gene* **23**, 331–341.

Uren, J. R. (1987). Cystathionine *β*-lyase from *Escherichia coli*. *Methods Enzymol* **143**, 483–486.

Van der Ploeg, J. R., Barone, M. & Leisinger, T. (2001). Expression of the *Bacillus subtilis* sulphonate–sulphur utilization genes is regulated at the levels of transcription initiation and termination. *Mol Microbiol* **39**, 1356–1365.

Wach, A. (1996). PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast* **12**, 259–265.

Weissbach, H. & Brot, N. (1991). Regulation of methionine synthesis in *Escherichia coli*. *Mol Microbiol* **5**, 1593–1597.

Yamagata, S. (1984). *O*-Acetylhomoserine thiolase of the fission yeast *Schizosaccharomyces pombe*: partial purification, characterization, and its probable role in homocysteine biosynthesis. *J Biochem* **96**, 1511–1523.

Yamagata, S. (1987). *O*-Acetyl-L-serine-*O*-acetyl-L-homoserine thiolase from *Saccharomyces cerevisiae*. *Methods Enzymol* **143**, 478–483.

Yamagata, S. (1989). Roles of *O*-acetyl-L-homoserine thiolases in micro-organisms. *Biochimie* **71**, 1125–1143.

Yamagata, S. & Takeshima, K. (1976). *O*-Acetylserine and *O*-acetylhomoserine thiolase of yeast. Further purification and characterization as a pyridoxal enzyme. *J Biochem* **80**, 777–785.

Yocum, R. R., Perkins, J. B., Howitt, C. L. & Pero, J. (1996). Cloning and characterization of the *metE* gene encoding *S*-adenosylmethionine synthetase from *Bacillus subtilis*. *J Bacteriol* **178**, 4604–4610.