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

Sandrine Auger, W. H. Yuen, Antoine Danchin and Isabelle Martin-Verstraete

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-acetylsereine or O-acetylhomoserine to produce cysteine or homocysteine, respectively. These reactions are catalysed by an O-acetylserine thiolase (EC 4.2.99.8) (Kredich, 1996), or by an O-acetylhomoserine thiolase (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.,...
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 & Bro, 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 yjcl (now metI) and yjcf (now metC) genes products in the biosynthesis of methionine in B. subtilis. We also demonstrated that the S-box sequence, which is located upstream of the metl 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 KH$_2$PO$_4$, 44 mM KH$_2$PO$_4$, 0.3 mM trisodium citrate, 5 mM MgCl$_2$, 0.5% glucose, 50 mg L-tryptophan 1−3, 22 mg ferric ammonium citrate 1−3, 0.1% L-glutamine) supplemented with a sulfur source as stated: 1 mM K$_2$SO$_4$, 1 mM L-methionine, 1 mM L-cysteine, 0.5 mM L-cystathionine, 0.1 mM L-cystathionine. Antibiotics were added at the following concentrations when

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**Fig. 1.** Biosynthetic pathways of sulfur-containing amino acids. Thiolation pathways: 2, O-acetylserylne; 3, O-acetylserylne thiolyase. Transsulfuration pathways: 4, cystathionine γ-synthase; 5, cystathionine β-lyase. Reverse transsulfuration pathway: 6, cystathionine β-synthase; 7, cystathionine γ-lyase. Other steps: 1, serine transacytase; 8, homoserine transacytase; 9, methionine synthase. Most Gram-negative bacteria use O-succinylhomoserine instead of O-acetylserylne.
To complement the Construction of plasmids and strains.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Source†</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td><strong>metB1 lac-3 (or lacY1)</strong> galK2 galT22 supE44 bsdR Rif**</td>
<td>I. Saint-Girons‡</td>
</tr>
<tr>
<td>WA802</td>
<td><strong>metC162::Tn10 rph-1</strong></td>
<td>E. coli Genetic Stock Center</td>
</tr>
<tr>
<td>CAG18475</td>
<td></td>
<td>Novagen</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F' ompT bsdS* (r6 m4) gal dcm (DE3)</td>
<td></td>
</tr>
</tbody>
</table>

| B. subtilis |                                      |           |
| 168         | *trpC2                                | Laboratory stock |
|            | **trpC2 metC::lacZ cat ΔmetC**         |            |
| BSIP1140    | **trpC2 amyE::(pΔmetI–lacZ cat)**     | pDIA5509 → 168 |
| BSIP1142    | **trpC2 amyE::(pΔmetI–lacZ cat)**     | pDIA5510 → 168 |
| BSIP1143    | **trpC2 metI::spc**                   | pDIA5517 → 168 |
| BSIP1153    | **trpC2 amyE::(pΔmetI–lacZ cat)**     | pDIA5524 → 168 |
| BSIP1154    | **trpC2 amyE::(pΔmetI–lacZ cat)**     | pDIA5525 → 168 |
| BSIP1162    | **trpC2 amyE::(pΔmetI–lacZ cat)**     | pDIA5540 → 168 |
| BSIP1163    | **trpC2 amyE::(pΔmetI–lacZ cat)**     | pDIA5541 → 168 |
| BSIP1164    | **trpC2 metI::spc**                   | pDIA5547 → 168 |
| BSIP1179    | **trpC2 metI::spc amyE::(pxyl metl cat)** | pDIA5544 → BSIP1143 |
| BSIP1184    | **trpC2 metI::spc amyE::(pxyl metl cat)** | pDIA5553 → BSIP1164 |
| BSIP1229    | **trpC2 Δ(Sbox–metC)::apbA3**         | This study |
| BSIP1300    | **trpC2 amyE::(pxyl metl–lacZ cat)**  | pDIA5600 → 168 |
| BSIP1301    | **trpC2 metl::spc amyE::(pxyl metl cat)** | pDIA5553 → BSIP1164 |
| BSIP1302    | **trpC2 amyE::(pΔmetI–lacZ cat)**     | pDIA5571 → 168 |

* cat, pC194 chloramphenicol acetyltransferase gene; *apbA3*, 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 1⁻¹ (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 I containment, agreement no. 2735).

The loss of amylase activity was detected as described by Stillke 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 37 °C.

The mean values of at least three independent experiments are presented. Standard deviations (SD) 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), Tag 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 dyeedge 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 *metl* (ycjL) and *metC* (ycjJ) genes were expressed under the control of a lac promoter in pH315 (Arantès & Lereclus, 1991). The coding sequences of *metl* (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'-BamHI site or a 3'-EcoRI site. These fragments were inserted between the BamHI and EcoRI sites of pH315.

*Metl* (nucleotides −55 to +170) and *metC* (nucleotides +1414 to +2975) DNA fragments were amplified by PCR using primers that introduced a BamHI or EcoRI restriction site at the 5'- or 3'-end. These fragments were inserted into pH101 (Ferrari et al., 1983), resulting in plasmids pH101metl and pJH101metC. A SmaI-restricted spectinomycin-resistance cassette (Murphy, 1985) was cloned into the *AgeI* site of blunt-ended pJH101metl and into the *MluI* site of pJH101metC, resulting in pDIA5517 and pDIA5547, respectively. These plasmids, linearized by *SacI*, were used to transform B. subtilis 168. The *metl* 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 *metl* and *metC* mutants with the *metl* and *metC* genes. The complete coding sequences of *metl* (nucleotides +159 to +1373) and *metC* (nucleotides +1127 to +2543) were amplified by PCR. A SpeI or BamHI restriction site was created at the 5'- or 3'-end of the fragments. These DNA fragments were inserted into the SpeI and BamHI sites of pX, producing pDIA5544 and pDIA5553. These plasmids were then used to transform different B. subtilis mutants, leading to the integration of the *metl* or *metC* gene at the *amyE* locus (Table 1).
then combined: the metl 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 metl 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–metC 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 Galton & 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). [γ-$^{32}$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 metl gene (Fig. 3). Northern blots were analysed and quantified with the Image Master 1D software (Amersham).

Overexpression of Metl. The metl gene (nucleotides +210 to +1340), amplified by PCR using primers introducing a 5′-NdeI and a 3′-XhoI site, was cloned into the pET20b+ vector (Novagen) which had been digested by NdeI and XhoI. The resulting plasmid, pET20b+metl, was transformed into E. coli WA802 (metB1), which carries the T7 RNA polymerase gene on pGPl-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 metl gene. The pET20b+metl 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 metl 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 thiolase 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,
except that cysteine was replaced with 8 mM Na$_2$S. The amount of l-homocysteine formed was determined by the nitroprusside reaction (Yamagata, 1987). Cystathionine $\beta$-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

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

Cystathionine $\gamma$-synthase, cystathionine $\beta$-lyase, cystathionine $\gamma$-lyase and O-acetylhomoserine thiolase belong 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]-X$_r$-[STAGC]-[STAGC]-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, yjcl, yjcJ and yrhB, encoding proteins with similar signature patterns. Yjcl, YjcJ and YrhB are proteins of 373, 390 and 379 aa, respectively. A multiple alignment of these polypeptides with the E. coli cystathionine $\gamma$-synthase (MetB) and cystathionine $\beta$-lyase (MetC) enzymes showed that the yjcl, 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_{660} 0.1 in M63 medium with 1 mM sulfate or 1 mM sulfate plus 1 mM methionine as sulfur source. OD_{660} was measured after 12 h growth at 37 °C.

<table>
<thead>
<tr>
<th><em>E. coli</em> strain (relevant genotype)</th>
<th>Plasmid</th>
<th>OD_{660} after 12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sulfate</td>
</tr>
<tr>
<td>WA02 (<em>metB1</em>)</td>
<td>pHT315</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>pHT315 <em>yjcI</em></td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>pHT315 <em>yjcJ</em></td>
<td>0.14</td>
</tr>
<tr>
<td>CAG18475 (<em>metC162::Tn10</em>)</td>
<td>pHT315</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>pHT315 <em>yjcI</em></td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>pHT315 <em>yjcJ</em></td>
<td>2.20</td>
</tr>
</tbody>
</table>

**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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Sulfate (1 mM)</th>
<th>Cysteine (0.5 mM)</th>
<th>Cystathionine (0.1 mM)</th>
<th>Homocysteine (1 mM)</th>
<th>Methionine (1 mM)</th>
<th>Doubling time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>168</td>
<td><em>yjcI::spc</em></td>
<td>48</td>
<td>57</td>
<td>50</td>
<td>53</td>
<td>55</td>
<td>5</td>
</tr>
<tr>
<td>BSIP1143</td>
<td><em>yjc::spc amyE::pxyl</em></td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>63</td>
<td>55</td>
<td>5</td>
</tr>
<tr>
<td>BSIP1179</td>
<td><em>yjc::spc amyE::pxylJ</em></td>
<td>56</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BSIP1184</td>
<td><em>yjc::spc amyE::pxyl</em></td>
<td>NG</td>
<td>ND</td>
<td>105</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BSIP1164</td>
<td><em>yjc::spc</em></td>
<td>55</td>
<td>57</td>
<td>ND</td>
<td>63</td>
<td>54</td>
<td>5</td>
</tr>
<tr>
<td>BSIP1301</td>
<td><em>yjc::spc amyE::pxylJ</em></td>
<td>58</td>
<td>ND</td>
<td>60</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

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 pH3T15, under the control of the lac promoter. The plasmids pH3T15, pH3T15*yjcI* and pH3T15*yjcJ* were introduced into the *E. coli* mutants WA802 (*metB1*) and CAG18475 (*metC162::Tn10*) (Table 1). In a minimal medium with sulfate as sulfur source, pH3T15*yjcI* restored the growth of the *metB* mutant, whereas pH3T15*yjcJ* allowed the growth of the *metC* mutant (Table 2). pH3T15 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 yjcI and yjcJ loci of the B. subtilis chromosome. To avoid cystathionine β-lyase activity, using cystathionine as substrate, was observed in extracts of cells harbouring pET20b+ O-Acetylhomoserine thiolyase activity of YjcJ was tested in E. coli strain BL21(DE3). Crude extracts of BL21(DE3) transformed with pET20b+ yjcJ showed O-acetylhomoserine thiolyase [7 μmol homocysteine min−1 (mg protein)−1] and O-succinylhomoserine thiolyase [67 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 yjcI mutant (BSIP1229) by introducing pHT315yjcI into this strain. Growth experiments in liquid minimal medium showed that the yjcI gene restored the growth of the yjcI 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 pAmel’–lacZ and metC–lacZ fusions in the presence of different sulfur sources

<table>
<thead>
<tr>
<th>Sulfur source</th>
<th>β-Galactosidase activity [U (mg protein)⁻¹]</th>
<th>BSIP1142 metI::pAmel’–lacZ</th>
<th>BSIP1140 metC–lacZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfate (1 mM)</td>
<td>515</td>
<td>265</td>
<td></td>
</tr>
<tr>
<td>Methionine (1 mM)</td>
<td>100</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Sulfate (1 mM) + Methionine (1 mM)</td>
<td>45</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Cystathionine (0–1 mM)</td>
<td>430</td>
<td>NG</td>
<td></td>
</tr>
<tr>
<td>Cysteine (0–5 mM)</td>
<td>475</td>
<td>245</td>
<td></td>
</tr>
<tr>
<td>Homocysteine (1 mM)</td>
<td>340</td>
<td>330</td>
<td></td>
</tr>
</tbody>
</table>

* The DNA fragment of the metl 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 metl-specific probe revealed a single 2.5 kb transcript, which corresponds to the size of a bicistronic metIC mRNA (Fig. 3c).

**Identification of the metl promoter**

The DNA sequence of the metl 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 metl 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 σ70-dependent promoters.

To confirm the position of the promoter, a transcriptional fusion was constructed between the lacZ gene and the metl 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)metl’–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 metl’–lacZ fusion, indicating the existence of a single promoter.

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

The cis-acting elements required for the regulation of the metlIC operon are present in the pA(−58; +263)metl’–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, metl 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)metl–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)metl–lacZ and pD(−58; +140)metl–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)metl–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 metl 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 pAmetl–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 metl 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 metl 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 metl 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 metl-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 metl 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, yicI, yicJ and yrbB, 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-acetylaminoacetohomoserine is used as the substrate instead of O-succinylhomoserine (Kanzaki et al., 1986). Enzyme assays revealed that the Metl protein has cystathionine γ-synthase activity and could catalyse the synthesis of cystathionine from O-acetylaminoacetohomoserine and cysteine (Fig. 1). The complementation of the E. coli metB mutant by the B. subtilis metl 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. YrbB, a Metl/MetC-like protein, which could be implicated in methionine degradation, is not involved.

The phenotype of the metl mutant (Table 3) shows that the Metl protein is important for the second pathway. The Metl polypeptide also exhibited O-acetylaminoacetohomoserine thiolase activity. This enzyme catalyses the synthesis of homocysteine from sulfide and O-acetylaminoacetohomoserine. Complementation with the metl gene allowed the B. subtilis ΔmetIC 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 thiolase activity. O-Acetylhomoserine thiolases 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 thiolase 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 thiolase 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 thiolases of Leptospira meyeri (28 % similarity) and Saccharomyces cerevisiae (29.7 % similarity). In addition, O-acetylhomoserine thiolases 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 thiolases (Cherest et al., 1993).

In E. coli, the metC gene corresponds to a single transcriptional unit and the metB gene forms an operon with metE, which encodes a bifunctional aspartokinase–homoserine dehydratase (Greene, 1996). In B. subtilis, the metE 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.

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REFERENCES


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.


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


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