

# Identification of *yrrU* as the Methylthioadenosine Nucleosidase Gene in *Bacillus subtilis*

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## Abstract

Taking trimethoprim as the selective agent in the presence of thymine, we adapted to *Bacillus subtilis* a selection procedure depending on the peculiar organisation of the one-carbon metabolism. The corresponding pathways couple synthesis of thymine to tetrahydrofolate consumption as a substrate of the reaction mediated by thymidylate synthase, instead of being a co-enzyme as in the other reactions transferring one-carbon groups. Mutants obtained are thymidylate synthase deficient, and therefore auxotrophic for thymine. This provides positive selection in a first step for gene replacement by a thymidylate synthase cassette, and subsequently against its presence. For systematic recombination of mutations constructed *in vitro*, we used the property of *B. subtilis* to grow at high temperature, noting that the *thyB* gene product is inactive at 46°C, while the product of *thyA* remains active at this temperature. As the first step, we built up a recipient *thyA*<sup>-</sup> background, deleting the gene by *in situ* recombination. This method was used to investigate the function of the *yrrU* gene, which is presumably involved in a sulfur recycling pathway associated with polyamine biosynthesis. We showed that *yrrU* codes for a protein recycling methylthioadenosine, probably a nucleosidase. In addition we observed that *B. subtilis* can use methylthioribose as a sulfur source, and that it is an efficient sulfur scavenger.

**Key words:** sulfur salvage; S-adenosyl methionine; spermidine; putrescine; sequential gene disruption

## 1. Introduction

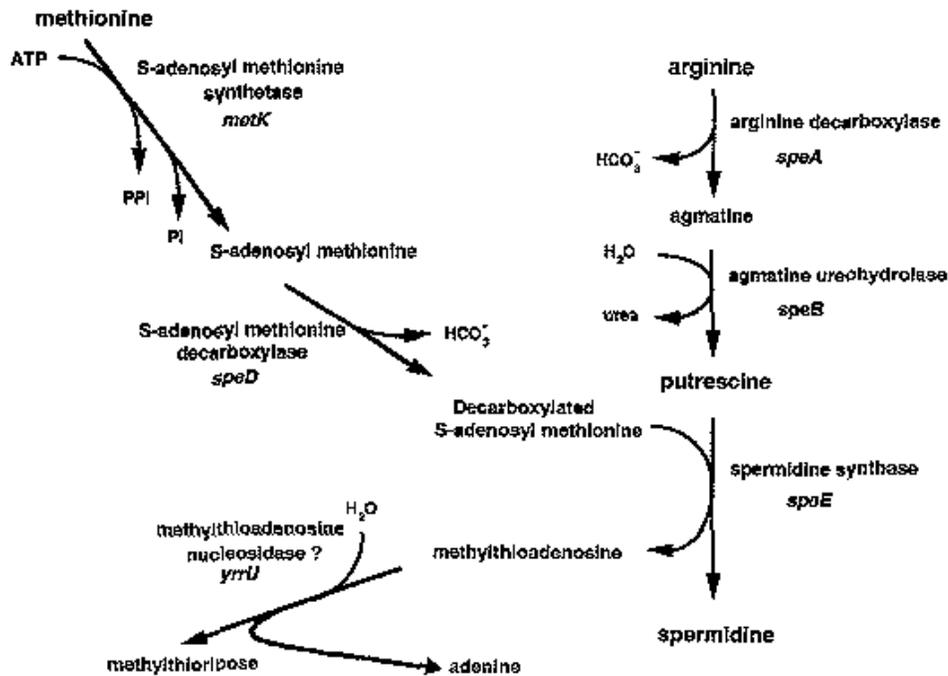
Genome programs have uncovered a variety of genes of yet uncharacterised function. In addition, they revealed that many genes form paralogous clusters. Such findings imply that, at least in some cases, a defect may be complemented partially or totally by a member of the set of paralogous counterparts. Consequently, the phenotype of a disrupted gene mutant might escape attention because a compensating paralog becomes expressed.<sup>1</sup> It may therefore be necessary to disrupt all paralogous genes in a family to reveal a clear-cut phenotype. Gene disruption is a simple way to understand the function of a gene. It is usually performed using *in vivo* recombination between a wild-type gene and an allele constructed *in vitro* by inserting an antibiotic resistance cassette between the intact borders of the gene. However, a problem immediately arises when one wishes to combine sequentially a set of disrupted mutants. This is because the number of available antibiotic resistance cassettes is limited. Furthermore, antibiotic resistance often interferes with the metabolic processes that one wishes to study.

For example, resistance to erythromycin often results in a general modification of all ribosomes in the cell.<sup>2,3</sup> This prompted us to reinvestigate methods that have been formerly developed to make sequential inactivation of genes in model organisms.

In the course of our reconstruction of polyamine metabolism in *B. subtilis*, we have shown that the *speE* (*ywhF*) gene coded for spermidine synthase.<sup>4</sup> This enzyme makes spermidine from putrescine and decarboxylated S-adenosyl methionine (dAdoMet). As shown in Fig. 1, the reaction yields the sulfur-rich molecule, methylthioadenosine (MTA) as a by-product. This molecule must somehow be recycled in order for the cell to recover the large amount of energy consumed for the construction of its ultimate precursor, S-adenosylmethionine (AdoMet). The fate of MTA has been studied in several organisms, but it is entirely known only in *Klebsiella aerogenes*.<sup>5–8</sup> It generally starts from the hydrolysis or phosphorolysis of MTA, yielding either methylthioribose (MTR) (in bacteria) or methylthioribose-1-phosphate (in eucaryotes or archaeobacteria). The enzyme responsible for this activity, a nucleosidase, has recently been identified in *Escherichia coli*, where it is coded by the *pfs* gene.<sup>9</sup> We decided to demonstrate our technique for sequential gene replace-

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**Figure 1.** Polyamine metabolism and the initial step of MTA recycling in *B. subtilis*. *yrrU* is involved in recycling; it is likely to code for a MTA nucleosidase. Other possible activities of YrrU, such as S-adenosyl homocysteine nucleosidase activity<sup>9</sup> have not been investigated in the present work.

ment by inactivating the *yrrU* gene, which could be responsible for MTA hydrolysis.

## 2. Materials and Methods

### 2.1. Strains, culture media and chemicals

*E. coli*, *B. subtilis* strains and plasmids used in this study are listed in Table 1. *E. coli* and *B. subtilis* were grown in Luria-Bertani (LB) medium, and/or minimal salt medium<sup>10</sup> with 27.7 mM glucose and either 15 mM L-glutamine or 15 mM ammonium as a nitrogen source. The medium was supplemented with 50 mg/liter L-tryptophan and 50 mg/liter thymine when necessary. For tests of various sulfur sources, a sulfur-free medium was used. This medium was as described above with slight modifications: L-glutamine was used exclusively as the nitrogen source and MgSO<sub>4</sub> was replaced by MgCl<sub>2</sub> at the same concentration (2 mM); each sulfur source was added at a 2 mM concentration, with the exception of methylthioribose (MTR) which was used at a concentration of 0.2 mM. For growth on plates, the same sulfur-free basal medium was supplemented with agar or agarose (1.5%) and with sulfur sources as above. When paper disks were used, 10  $\mu$ l of MTA (100 mM stock solution) or MTR (200 mM stock solution) was applied onto the disks. When included, antibiotics were added at the following concentrations: ampicillin, 100 mg/liter;

trimethoprim, 10 mg/liter. Bacteria were grown at 37°C or 46°C as indicated.

*E. coli* and *B. subtilis* were transformed as described by Sambrook *et al.*<sup>11</sup> and Kunst and Rapoport,<sup>12</sup> and transformants were selected on appropriate LB or minimal medium plates.

MTR was prepared from MTA (Sigma, D5011) by acid hydrolysis as described by Schlenk *et al.*<sup>13</sup>

### 2.2. Molecular genetics techniques

Standard procedures were used for extracting plasmids from *E. coli*.<sup>11</sup> Restriction enzymes and T4 DNA ligase were used as specified by manufacturers. DNA fragments used for cloning experiments were prepared by PCR using *Pfu* Turbo DNA polymerase (Stratagene) (for *thyA* deletion) or AccuTaq LA DNA polymerase (Sigma). Amplified fragments were purified using the QIAquick PCR Purification Kit from Qiagen.

To construct the *thyA* deletion strain (BSIP7001), two DNA fragments, one upstream from the *thyA* gene (nucleotides -490 to -92 relative to the translational start point) and the second one downstream from the *thyA* gene (nucleotides +79 to +535 relative to the stop codon) were amplified by PCR. For the upstream fragment, primers introducing an *EcoRI* recognition site at the 5' end and a *HindIII* recognition site at the 3' end were used. For the downstream fragment, one that introduced

**Table 1.** Bacterial strains and plasmids used or created in this study.

Strain or plasmid	Genotype or description	Source or reference
Strains		
<i>Escherichia coli</i>		
XL1-Blue	K12 <i>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac<sup>-</sup> F'[proAB<sup>+</sup> lacI<sup>q</sup> lacZΔM15 Tn10(tet<sup>R</sup>)]</i>	Laboratory collection
<i>Bacillus subtilis</i>		
168	<i>trpC2</i>	Spizizen <sup>40</sup>
BSIP7001	<i>trpC2 ΔthyA</i>	This work
BSIP7002	<i>trpC2 ΔthyA yrrU::thyA</i>	This work
BSIP7003	<i>trpC2 ΔthyA yrrU::pfs</i>	This work
Plasmids		
pUC19	cloning vector, Amp <sup>R</sup>	Yanisch-Perron <sup>41</sup>
pDIA5397	pUC19Δ <i>thyA</i>	This work

a *Hind*III recognition site at the 5' end and one that introduced a *Bam*HI site at the 3' end were employed. PCR products were ligated and inserted into the *Eco*RI and *Bam*HI sites of pUC19 (Boehringer Mannheim) creating plasmid pDIA5397. Prior to transformation, this plasmid was linearised at its unique *Sca*I site. Transformants were recovered on minimal medium plates (with ammonium as a nitrogen source) supplemented with thymine and trimethoprim at 46°C. Complete deletion of the gene was obtained by a double crossover event. Clones were checked by PCR using two primers located at 218 to 197 bp upstream and 234 to 255 bp downstream of *thyA*, respectively.

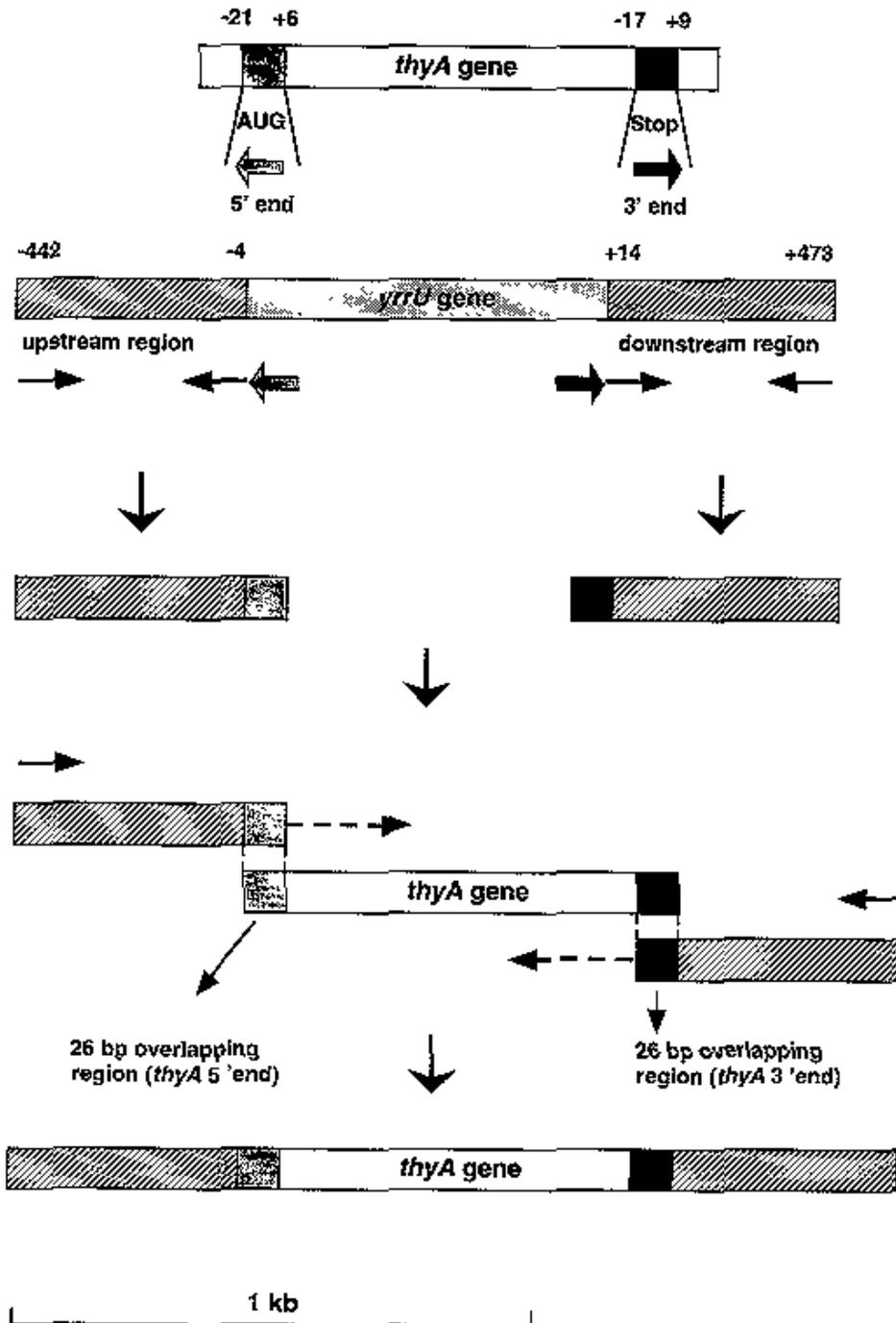
To construct a *yrrU<sup>-</sup>* strain (BSIP7002) and a *yrrU::pfs* substituted strain (BSIP7003), the four primer PCR procedure was used as described by Wach.<sup>14</sup> In brief, in a first step the *thyA* (or *pfs*) gene was amplified from chromosomal DNA and was subsequently used as a template (Fig. 2). To obtain a deletion cassette, two 440–460 bp fragments from the regions upstream (nucleotides –442 to –4 relative to the translational start point of *yrrU*) and downstream (nucleotides +14 to +473 relative to the *yrrU* stop codon) of *yrrU* were amplified in such a way that 26-bp fragments were introduced at one of their ends (see Fig. 2). These fragments were, in the case of the *yrrU::thyA* mutant (BSIP7002), homologous to the 5' *thyA* end (nucleotides –21 to +6 relative to the translational start point of *thyA*) and the 3' *thyA* end (nucleotides –17 to +9 relative to the *thyA* stop codon), respectively. In the case of the *yrrU::pfs* substituted mutant (BSIP7003), these fragments were homologous to the 5' *pfs* end (nucleotides –3 to +24 relative to the translational start point of *pfs*) and the 3' *pfs* end (nucleotides –15 to +12 relative to the *pfs* stop codon), respectively. In the next step, three DNA fragments were mixed: the *yrrU* upstream region with a 26-bp *thyA* (or *pfs*) fragment at its 3' end, the *yrrU* downstream region

with a 26-bp *thyA* (or *pfs*) fragment at its 5' end, and the complete *thyA* gene (*pfs* gene) surrounded by two 26-bp fragments added in the upstream and downstream region of *yrrU* (see Fig. 2). The two *yrrU* fragments overlapping at one of the ends with the *thyA* gene (*pfs* gene) served as a long primer in PCR using *thyA* or *pfs* as a template. In this second PCR reaction, two external primers (5' upstream and 3' downstream primer) were added in the reaction mixture to increase amplification efficiency. The product corresponding to the two regions flanking *yrrU*, with the inserted *thyA* or *pfs* cassette in-between, was purified from the gel, and a PCR reaction was repeated to obtain a pure product. For the *yrrU* deletion strain construction, strain BSIP7001 (*ΔthyA* strain) was used as the recipient, and transformants were obtained by incubating at 46°C on minimal medium plates with ammonium as a nitrogen source (without thymine). Transformants were checked by PCR, using two primers located 27 to 4 bp upstream and 14 to 39 bp downstream of *yrrU*. For the *yrrU::pfs* substituted mutant, strain BSIP7002 (*ΔthyA yrrU::thyA* strain) was used as the recipient and transformants were selected as above, on plates supplemented with thymine and trimethoprim at 46°C. Transformants were checked by PCR with the same primers as for the *yrrU::thyA* strain.

### 3. Results

#### 3.1. Rationale for procedure

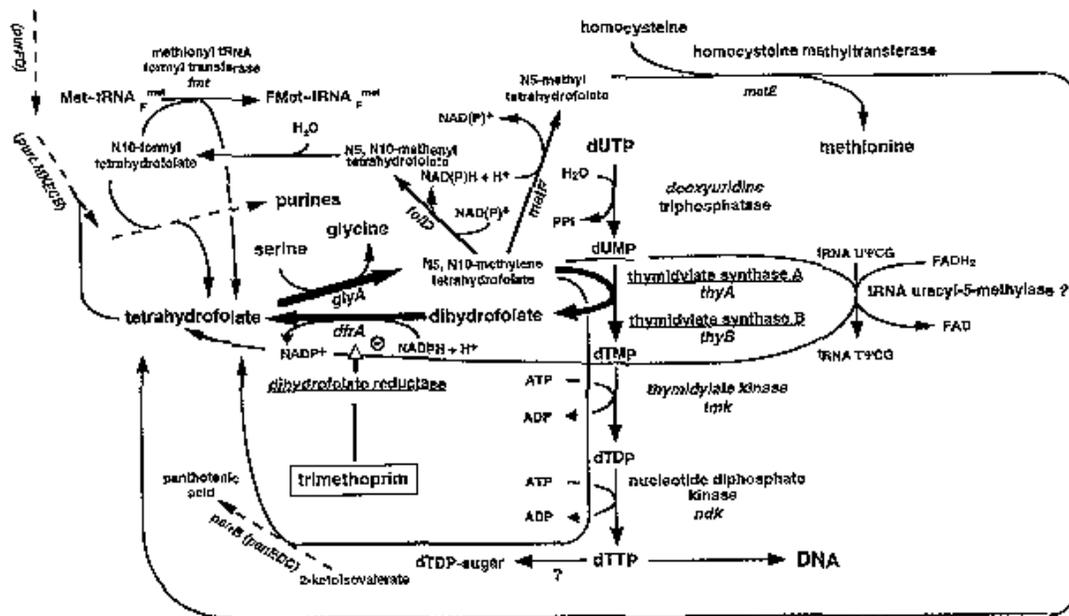
Gene inactivation or replacement is a process of mutagenesis. Obtaining desired mutants depends on whether one has an efficient screening or, better, selection procedure. This is usually performed using inhibitors affecting the growth of wild-type cells. To inhibit the growth of a cell, a molecule must pass from the culture medium into the cell where it goes to a proper location. The inhibitor must affect some vital functions



**Figure 2.** Gene replacement with the *thyA* cassette, using the four-primer PCR technique.<sup>14</sup> Construction of a *thyA* cassette bracketed by *B. subtilis* DNA segments 440–460 bp long allowed direct transformation and recombination for gene replacement *in situ*. For details, see Materials and Methods, and Results.

of the cell and stop growth. Furthermore, the effect of the inhibitor must be as specific as possible, so that a single target is generally hit. This will restrict the number of hypotheses and hence the culture conditions that

have to be tested. In order to ensure the application of same type of selection pressure, it is often useful to have procedures that use the same metabolic pathway for forward and backward selection. In *E. coli* or *Sac-*



**Figure 3.** One-carbon metabolism in *B. subtilis*. The tetrahydrofolate coenzyme is recycled in all reactions except those allowing thymidylate synthesis (the tetrahydrofolate-dihydrofolate cycle is shown by thick arrows). In this case each reaction turnover leads to dihydrofolate, and requires replenishment of the tetrahydrofolate pool by dihydrofolate reductase. The name of relevant enzymes (thymidylate synthases and dihydrofolate reductase) is underlined.

*Saccharomyces cerevisiae*, several metabolic systems are well suited for such selection procedures.<sup>15</sup> Lacroute and his co-workers have devised a means to sequentially replace genes in *S. cerevisiae*. This procedure makes use of the fact that the yeast is sensitive to 5-fluoroorotate (5-FOA) in the presence of uracil when orotidine monophosphate decarboxylase, an enzyme of pyrimidine metabolism, is active. In the first round of selection (on plates containing 5-FOA and uracil) one obtains orotidine monophosphate decarboxylase-deficient mutants as the majority of growing colonies.<sup>16</sup> These mutants are auxotrophic for uracil. Thus, in the second round of selection, omitting uracil or uridine from the medium will suffice for selecting those that have reverted to an orotidine monophosphate decarboxylase-positive phenotype.

We tried to apply this procedure to *B. subtilis*. Unfortunately, it was found that the bacteria were not sensitive enough to 5-FOA under standard growth conditions. We therefore used another strategy employing trimethoprim as a selective agent in the presence of thymine.<sup>15,17</sup> This selection procedure rests on the peculiar organisation of the one-carbon metabolism. In this metabolism, thymine synthesis is coupled to tetrahydrofolate consumption mediated by thymidylate synthase. However, instead of being a recycled co-enzyme as in the other reactions, tetrahydrofolate serves as a substrate of the reaction transferring one-carbon groups (see Fig. 3). Mutants thus obtained (at least in minimal medium) are thymidylate

synthase deficient, and therefore, auxotrophic for thymine.

The situation in *B. subtilis* is not as straightforward as in *E. coli*, the paradigm for this experiment. Indeed, because these bacteria harbour two thymidylate synthase genes, *thyA* and *thyB*,<sup>18</sup> finding the desired trimethoprim-resistant mutants in this organism is usually not possible. A second possible difficulty in the selection process is that the ribothymidine of tRNA (in the TΨCG loop) results from a tetrahydrofolate-mediated methylation (Schmidt,<sup>19</sup> see discussion below) and not from AdoMet methyl transfer as in *E. coli*. Using *thyA thyB* double mutant<sup>18,20</sup> Kiel *et al.*<sup>21</sup> have proposed a method for the selection of thymine auxotrophic mutants in *B. subtilis*. These authors used *thyP3*, the thymidylate synthase gene from the bacteriophage ϕ3T of *B. subtilis*, as the selective marker cassette. Unfortunately, the origin of the recipient strain, which is a spontaneous mutant (not a deletion), created a problem of recombination between the *thyA* gene and *thyP3* (we now know that *thyA* is likely to be of phage origin<sup>22</sup>). To generalise the method and to use it for systematic recombination of mutants constructed *in vitro*, we took advantage of the ability of *B. subtilis* to grow at high temperature.<sup>23</sup> It has been noted that the *thyB* gene codes for a thermostable product that shows no activity above 46°C while the product of the *thyA* gene is thermostable.<sup>18</sup> Therefore, when grown at high temperature in the presence

of trimethoprim and thymine, a significant proportion of growing colonies are devoid of thymidylate synthase. They, however, recover this enzyme activity at 37°C. This approach was used to build our recipient *thyA*<sup>-</sup> background. We first deleted the gene by *in situ* recombination with a DNA segment bracketing the *thyA* gene. This segment was constructed by fusing together the 5' and 3' ends of the PCR-amplified DNA fragments immediately upstream and downstream from the *thyA* gene (see Materials and Methods). This construction was introduced into *B. subtilis* 168 to give strain BSIP7001. As expected, this strain is a thymine auxotroph at 46°C.

The procedure subsequently requires the construction of a PCR product such that the gene to be deleted could be replaced with the functional *thyA* gene (see Materials and Methods, and Fig. 2). The *thyA*<sup>-</sup> strain BSIP7001 was transformed with the amplified DNA, selecting for recombinants that recovered prototrophy at the high temperature under conditions leading to inactivation of the *thyB* gene product. The second step consists of replacing the *thyA* gene by any chosen DNA fragment allowing the cells to grow at 46°C in the presence of trimethoprim and thymine (conditions where only the cells lacking ThyA activity can grow).

### 3.2. Inactivation of gene *yrrU*, and phenotype of recombinant

Using the SubtiList specialised database,<sup>24</sup> we looked for a counterpart of the *E. coli pfs* gene and found that a gene, *yrrU*, presumably in an operon (*yrrTUyrhABC*) could code for the appropriate activity. Employing our *thyA* background strain at 46°C, we replaced the *yrrU* gene with *thyA*, as depicted in Fig. 2. To test the phenotype of the *yrrU::thyA* mutant, we grew it on agar plates without a sulfur supplement in the presence of paper disks impregnated with MTA or its MTR derivative. Very slow background growth was detected on the plates, presumably due to hydrolysis of sulfate from the sulfato-carbohydrates present in agar. Conspicuous growth was observed around the disk containing MTR with both the *yrrU*<sup>+</sup> and the *yrrU*<sup>-</sup> strains. In contrast, when the disk was impregnated with MTA, growth was only observed with the wild-type strain. Moreover, the growth was significantly slower than that with MTR. This observation was substantiated by monitoring growth of the mutant and its wild-type parent in sulfur-free liquid medium supplemented with MTA or MTR (Table 2).

We constructed our gene replacement in such a way as to exchange a coding sequence (namely, that of *yrrU*) with another one (namely, that of *thyA*) without introducing other causes for transcription termination that we could identify for such an action. In this way we do not have to identify precisely the function of the genes located downstream from the replaced gene in the phenotype we study, such as the hydrolysis of MTA and the recycling

**Table 2.** Doubling time (minutes) in media<sup>a)</sup> containing various sulfur sources<sup>b)</sup>.

Strain <sup>c)</sup>	SO <sub>4</sub> <sup>2-</sup>	L-Met	Taurine	MTA	MTR
WT	90	90	570	120	90
BSIP7002	390	390	500	1440	300
BSIP7003	390	300	450	420	300

a) sulfur-free minimal medium.

b) all sulfur sources were added at 2 mM concentration, in exception of MTR (0.2 mM).

c) BSIP7002, *yrrU* mutant; BSIP7003, *yrrU::pfs* substituted mutant.

of MTR as a sulfur source. In this study, we confirmed that, as already noted in other organisms<sup>25-27</sup> and in contrast to *E. coli*, *B. subtilis* can utilise methionine as a sulfur source. Because the genes located downstream from *yrrU* are conceivably involved in sulfur metabolism, we looked for growth of the mutant in media where methionine serves as the sole sulfur source. The results were affirmative.

### 3.3. Replacement of *yrrU* by its presumably orthologous *E. coli* counterpart *pfs*

In order to go further, and test whether the inactivation of *yrrU* can be complemented by a known function, we put the *E. coli pfs* gene in the place of *yrrU*. A complete *pfs* coding sequence isolated from *E. coli* by PCR was used to replace the *thyA* gene inserted in *yrrU* (see Materials and Methods). The growth of the wild-type, the *thyA* insertion, and the *pfs* replacement mutants in liquid media were then compared. Results presented in Table 2 indicate that MTA serves as a sulfur source only in the wild-type and in the *pfs* replacement mutant. Some residual growth (generation time: 24 hr) can nevertheless be seen in the deletion mutant. MTR, on the other hand, remains a sulfur source in all cases. However, as can be seen in the data, it cannot restore the normal growth rate in the *yrrU::thyA* deletion, nor in its *pfs* replacement strain.

## 4. Discussion

### 4.1. Sulfur salvage and methylthioadenosine recycling in *B. subtilis*

The synthesis of spermidine consumes large amounts of energy. In addition, a molecule of AdoMet is used in the process, which also requires a great deal of energy in its formation. This includes the reduction of sulfur compounds to sulfide and complete hydrolysis of ATP.<sup>28</sup> Curiously, *E. coli* does not recycle the sulfur derivative of AdoMet, but hydrolyses MTA to MTR. For this activity *E. coli* uses a nucleosidase (encoded by *pfs* gene), as does *K. aerogenes*.<sup>5</sup> Since *E. coli* does not possess an MTR

kinase, it cannot recycle this molecule, that is excreted into the medium.<sup>29</sup> This can be related to the fact that *E. coli* cannot use methionine as a sulfur source either. Eucaryotes use a different pathway: They metabolise MTA directly to MTR-1-phosphate, using a nucleoside phosphorylase.<sup>30</sup> Archeobacteria appear to behave in the same way.<sup>31</sup> Indeed the starting point for MTR recycling is its phosphorylated form.<sup>5</sup> In contrast, *B. subtilis*, that lives in an environment generally poor in sulfur sources, has evolved efficient sulfur assimilation and salvage pathways. As we have shown in this work, this organism is able to metabolise MTA and MTR. This may be related to the fact that *B. subtilis* can use methionine as a sulfur source, which has also been substantiated in the present study. In addition, we have observed that *B. subtilis* is an efficient sulfur scavenger. Its ability to grow on agar and even agarose (data not shown) plates demonstrates that it must code for a secreted sulfato-carbohydrate sulfatase. Growth with MTA requires the presence of the *yrrU* gene, that is likely to be a MTA nucleosidase since it is most similar to the *E. coli pfs* gene product, and much less to known nucleoside phosphorylases, including the MTA phosphorylase of *Sulfolobus solfataricus*.<sup>31</sup> In particular, several conserved residues are specific to nucleosidases, as well as the absence of a gap (or a shorter gap) in the alignment, that appears to be specific for phosphorylases (as are other conserved residues) (Fig. 4).

As shown in Table 2 the *yrrU* deletion mutant (as well as its *pfs* replacement) does not grow as well as the wild-type when MTR is added as the sulfur source. This suggests that, upstream of the MTA nucleosidase activity, some product accumulated in the *yrrU* mutant is inhibitory to growth. MTA—which is probably accumulated under these circumstances—could inhibit major cell activities (in particular essential methylation steps<sup>32,33</sup>). Since the substitution of *yrrU* by *pfs* was limited to the coding sequence, the fact that *pfs* did not relieve this growth defect could be due to the fact that this heterologous gene is not properly expressed in *B. subtilis*, presumably because of its foreign codon preference (*B. subtilis* and *E. coli* have very distinct codon preferences<sup>34</sup>). Some residual growth with MTA persisted in the *yrrU* mutant. This may be due to hydrolysis of the MTA molecule, or to the induction of other sulfur salvage processes in *B. subtilis*. In this respect, it was noted that the *yrrU* mutant appeared to grow much better on agar plates without an added sulfur source than its wild-type parent does. This indicates that the MTA salvage pathway is normally active in *B. subtilis* and that it regulates the processes for acquiring sulfur from the medium. Such results corroborate the observation that *B. subtilis* normally lives in a sulfur-poor environment. Finally, we observed that growth with MTA is slower than with MTR. This suggests that in contrast to MTR, MTA permeation in *B. subtilis* is not very efficient. The negative inside electric potential of the cell might allow slow diffusion of a

slightly positively-charged compound such as MTA. The co-existence of a low concentration of the enzyme due to poor translation and low concentration of the substrate would limit growth in spite of the low  $K_M$  of the enzyme ( $0.31 \mu\text{M}^{35}$ ).

#### 4.2. Trimethoprim resistance in *Bacillus subtilis*

As we have briefly discussed, the process for obtaining thymine auxotrophs in the presence of trimethoprim is much more complicated in *B. subtilis* than in *E. coli*. The mechanism underlying this process is that the cell must produce tetrahydrofolate for growth, and that synthesis of each thymine residue oxidises one tetrahydrofolate to one dihydrofolate. Therefore, besides the need for doubling the tetrahydrofolate pool at each generation, the cell also requires that this pool be renewed continuously while constituting its dTTP pool. dTTP is used for DNA synthesis. In some bacteria (we did not find data about *B. subtilis* in this respect), it is also used to create a pool of dTDP-carbohydrates necessary for the construction of the cell wall and the outer layers of the cell envelope. In a cell such as a *B. subtilis* bacterium, the concentration of one single molecule (if one can speak of a concentration in that case) is ca. 1 nM. For a chromosome comprising ca. 4 million base pairs of average A+T content, this corresponds to an equivalent concentration of ca. 2 mM in thymine. If it exists, the pool of dTDP-carbohydrates would be in the same concentration range. Maintaining these pools requires constant replenishing of the tetrahydrofolate pool from dihydrofolate produced by the action of thymidylate synthase. As stated earlier, two such enzymes exist in *B. subtilis*, making it necessary to apply selection pressure allowing only one of the enzymes to be active at a given temperature (Fig. 3). Another activity, specific to *B. subtilis*, might complicate the picture. In many Gram-positive bacteria, synthesis of the ribothymidine in the T $\Psi$ CG loop of tRNA molecules is not, as in *E. coli*, performed by an AdoMet-dependent methylase (coded by the *trmA* gene). It is the result of a process similar to that of dTMP synthesis, with methylene-tetrahydrofolate as a cofactor.<sup>19</sup> Because the concentration of tRNA molecules is quite significant (one order of magnitude higher than the concentration of the thymidine pools discussed above and perhaps even significantly higher), this would make this reaction a major contributor of the trimethoprim effects in Gram-positive bacteria. Presumably however, this reaction does not oxidise tetrahydrofolate to dihydrofolate as in thymidylate synthase, but uses FADH<sub>2</sub> as the reducing agent.<sup>36</sup> It has nevertheless been shown that trimethoprim-treated *B. subtilis* strains possess unmodified tRNA molecules with U $\Psi$ CG instead of T $\Psi$ CG in the tRNA T-loop, and that, even under normal growth condition, all tRNA molecules are not modified.<sup>19</sup> Lack of methylation was further shown to permit growth of the



wild-type in media supplemented with the one-carbon metabolites and trimethoprim (therefore with cells lacking both T in tRNAs and formylation of the initiator methionine).<sup>37,38</sup> This was in contrast with the situation in *E. coli*, that indicated strong coupling between the one-carbon metabolism and macromolecular syntheses in bacteria.<sup>39</sup> Further experiments will clarify the role of the one-carbon pool and the sulfur and nitrogen metabolisms in integrating macromolecular syntheses with the intermediary metabolism at a global level in *B. subtilis*.

#### 4.3. Conclusion

Beside demonstrating that the *thyA* gene can be used as both a selection and counterselection agent to construct multitudinously mutated *B. subtilis*, we have identified the first step of MTA recycling, leading to the synthesis of MTR, allowing sulfur salvage in this organism. This has important consequences for the metabolism of polyamines and sulfur, particularly because it appears that MTR is a good sulfur source in *B. subtilis*. Moreover, this allowed us to uncover important possibilities for cross-talk between one-carbon and nitrogen and sulfur metabolisms in this organism. Further experiments will be needed to explore these points.

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