

Research article

MtnK, methylthioribose kinase, is a starvation-induced protein in *Bacillus subtilis*

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Abstract

Background: Methylthioadenosine, the main by-product of spermidine synthesis, is degraded in *Bacillus subtilis* as adenine and methylthioribose. The latter is an excellent sulfur source and the precursor of quorum-sensing signalling molecules. Nothing was known about methylthioribose recycling in this organism.

Results: Using trifluoromethylthioribose as a toxic analog to select for resistant mutants, we demonstrate that methylthioribose is first phosphorylated by MtnK, methylthioribose kinase, the product of gene *mtnK* (formerly *ykrT*), expressed as an operon with *mtnS* (formerly *ykrS*) in an abundant transcript with a S-box leader sequence. Although participating in methylthioribose recycling, the function of *mtnS* remained elusive. We also show that MtnK synthesis is boosted under starvation condition, in the following decreasing order: carbon-, sulfur- and nitrogen-starvation. We finally show that this enzyme is part of the family Pfam 01633 (choline kinases) which belongs to a large cluster of orthologs comprising antibiotic aminoglycoside kinases and protein serine/threonine kinases.

Conclusions: The first step of methylthioribose recycling is phosphorylation by MTR kinase, coded by the *mtnK* (formerly *ykrT*) gene. Analysis of the neighbourhood of *mtnK* demonstrates that genes located in its immediate vicinity (now named *mtnUVWXYZ*, formerly *ykrUVWXYZ*) are also required for methylthioribose recycling.

Background

Starvation for essential metabolites results in the expression of many proteins, often of still unknown function, some of which related to quorum-sensing. In the course of our study of sulfur metabolism in bacteria we have witnessed that expression of many genes was induced

when cells were deprived of sulfur [1]. In particular, upon entry into the stationary growth phase (which is often the consequence of starvation in one of the major cell metabolite supplies: carbon, nitrogen or phosphorus), we observed that polyamine biosynthesis was much affected, in parallel with the expression of S-adenosylme-

thionine decarboxylase [2]. This prompted us to investigate the fate of the products of this important enzyme.

Aminopropyl transfer, during polyamine metabolism, yields methylthioadenosine, which is split into adenine and methylthioribose by the *mtnA* gene (*yrrU*) [3]. This molecule is excreted in *Escherichia coli* [4], and degraded by oxidation steps in *Klebsiella pneumoniae* [5]. In Eukarya, its fate is not known except for parasites [6] and in part in plants [7] and mammals [8], and, because it appears to differ in different organisms, pharmaceutical companies have endeavoured to use analogues as drugs [9]. Nothing is known in other organisms. We have established that MTR is an excellent sulfur source in *Bacillus subtilis* [3]. Scanning the genome sequence however did not reveal obvious similarities with known pathways. Using a toxic analogue of MTR and transposon mutagenesis we screened for resistant mutants. This led us to identify the *ykrT* gene as a major bottleneck in MTR metabolism, and to identify it as the methylthioribose kinase gene (now named *mtnK*). Further study of the gene uncovered an unusual expression pattern under starvation conditions, discussed in the present article.

Results

Transposon mutagenesis

Methylthioribose (MTR) can be used by *B. subtilis* as the sole sulfur source. However, the way by which the sulfur moiety is recycled remains unknown. To elucidate this pathway, we used an analogue of MTR, trifluoromethylthioribose (3F-MTR), which has been assayed as a possible drug lead for killing pathogens [10]. After transposon mutagenesis (see Materials and Methods), eleven 3F-MTR resistant clones were obtained (located in genes *ykrW*, *ykrY* and *ykrT*). Among them, one mutant carried the transposon insertion in the putative promoter region of the *ykrT* gene (strain BSHP7035). This insertion was situated 73 bp upstream of translational start point and was comprised within the regulatory S-box structure [11]. Analysis of the remaining ten mutants will be the object of a separate publication (see Conclusion). Inactivation of gene *ykrT* resulted in complete loss of MTR utilization, although some revertant clones able to grow very poorly on MTR appeared after few days incubation. *In silico* analysis of the protein sequence revealed that it contained a sequence (PFAM CD motif 01633) typical of choline/ethanolamine kinases (Fig. 1 and [http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF01633]). We therefore explored whether as in *K. pneumoniae*, the first step of MTR catabolism would be phosphorylation of the ribose moiety.

MtnK (YkrT) is a methylthioribose kinase

Before investigating the involvement of YkrT in MTR metabolism we reanalysed its sequence, as predicted in SubtiList [http://genolist.pasteur.fr/SubtiList/]. The region preceding the putative ATG start site revealed the presence of three ATGs in a row. The putative ribosome binding site GGAGGT, should be located at least 5 nt and preferably 7–12 nt before the start site [12,13]. We therefore propose that the start of the protein is the third ATG in this sequence, and not the first one:

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tttacggccacatattaattaattacataattGGAGGTt atg atg ATG
gga gtc aca
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Sequence comparison with proteins present in bacterial genome databases [14] revealed, in addition to many genes annotated as choline/ethanolamine kinases with weak similarities (Fig. 1), nine proteins which displayed high similarity with YkrT: in *K. pneumoniae* (a protein annotated as a MTR kinase in the unpublished entry: Entrez Protein AAG43573), in *Bacillus stearothermophilus*, in two similar sequences of *Bacillus anthracis*, in *Treponema denticola*, in *Sinorhizobium meliloti*, in *Mesorhizobium loti* and in *Arabidopsis thaliana* (Fig. 2). The similarity with the sequence of *B. stearothermophilus* further substantiated our identification of the third ATG codon as the translation start site in *B. subtilis*.

Because it is well known that *in silico* function identification can be very misleading [15], we looked for a biochemical identification of the activity. Using protocols derived from the work of Riscoe and co-workers [16], we set up a cell-free biochemical assay, comparing the wild type strain, a disrupted conditional mutant (BFS1850), and this same mutant grown in the presence of IPTG (see also below), using radioactive ATP as the phosphate donor. As shown in Fig. 3, we found activity only in the wild type. Radioactive MTR-1-P was detected in the wild type when adding exogenous MTR, but not in its absence, nor in the *mtnK* of the enzyme for MTR has been mutant. The K_M approximately evaluated to be ca 60 μ M (Fig. 4). We also found that in the preparations containing 5% glycerol, the wild type contained glycerol-1-P, whereas the mutant did not, showing that MtnK can phosphorylate glycerol (data not shown).

mtnK and mtnS form an operon

Sequence analysis of the chromosome region of *mtnK* shows that this gene is situated between two predicted transcriptional terminators together with the adjacent *ykrS* gene. The *ykrS* gene is separated by only 7 bp from *mtnK*. This suggests that both genes could be co-transcribed. The transposon insertion yielding 3F-MTR resistance was identified in the promoter region of the *mtnK ykrS* genes. This could possibly abolish the expres-



Figure 1
 Comparison of MtnK with the first eight proteins listed in the choline kinase family Pfam 01633. Identical residues in the family 01633 are indicated by a "=" sign, while conserved residues are indicated by a "+" sign (amino acid families are: ASTPG, MLIV, HKR, WYF, DNEQ, and C). When these same residues are also conserved in MtnK they are colored: red for identical residues, blue for conserved residues.

action of both genes, as we were unable to tentatively identify *in silico* any putative promoter for *ykrS* gene alone. To explore this question, we used strain BFS1850 (*ykrT::lacZ*) which allows the expression of the downstream gene under the control of the IPTG inducible Pspac promoter. The BFS1850 strain was assayed for its ability to use MTR as the sole sulfur source in the presence or in the absence of IPTG. In both cases this strain failed to grow on MTR. Furthermore, expressing the *ykrS* gene from Pspac did not change the outcome of the biochemical experiment with inactivated *mtnK*, showing that *ykrS* does not directly participate in the MTR kinase activity (Fig. 3). As a further exploration of the *ykrS* gene role, we constructed a strain deleted of *ykrS* alone (BSHP7010). This strain was also unable to grow with MTR as the sulfur source. These results show that both *mtnK* and *ykrS* are implicated in the MTR recycling pathway (we propose therefore to rename *ykrS* *mtnS*), but that MtnK alone is involved in the phosphorylation step of the substrate.

Subsequently, the RNA synthesis in this region was analysed by RT-PCR in cells growing exponentially in minimal medium. This experiment confirmed that *mtnK* and

mtnS were transcribed together (data not shown). Because we found that disruptants of the upstream *ykrU* (*mtnU*) gene (an other adjacent gene of yet unidentified function – hydrolase/nitrilase-like), were unable to grow with MTR as the sulfur source, we further investigated by RT-PCR whether *mtnK* was co-transcribed with the *mtnU* gene. These experiments showed that there was no co-transcription, in line with the observation that *mtnU* is separated from *mtnK* by a transcriptional terminator (data not shown).

Expression of the *mtnK* gene

Using a *mtnK::lacZ* fusion (strain BFS1850) we studied the behaviour of the gene in a variety of environmental conditions. In the standard growth conditions with sulfate as sulfur source, the expression of *mtnK* was high, and very stable during growth, decreasing from 1700 U/mg of protein in the exponential growth phase to 1200 U/mg of protein in the stationary growth phase. Although the *mtnK* *mtnS* operon is a member of the S-box regulon, no significant factor of regulation by methionine availability could be observed under our growth conditions, when sulfate was replaced by methionine as sole sulfur source (30% variation, see Table 1). Under

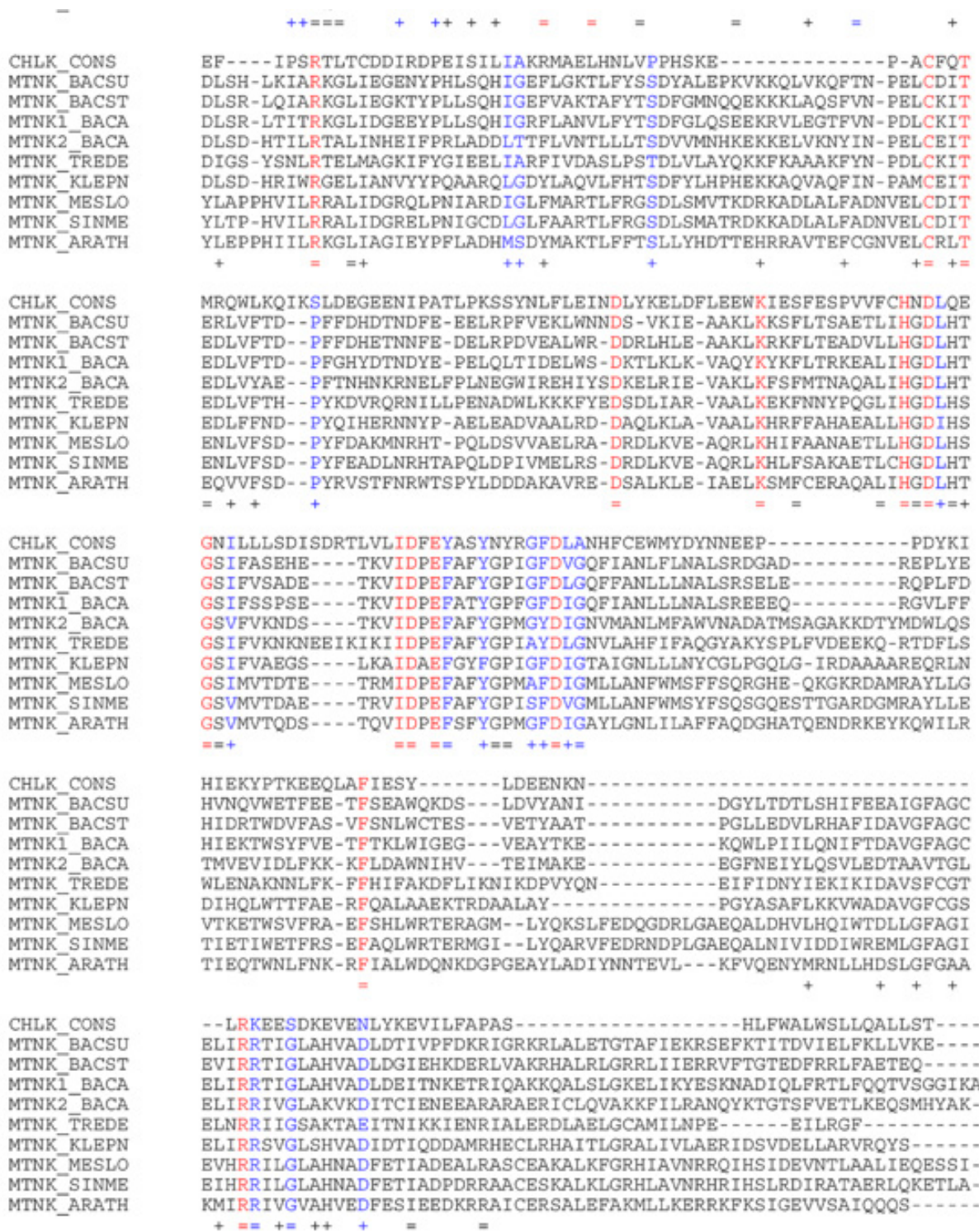


Figure 2
 Alignment of the eight putative MtnK protein present in the genome libraries with *B. subtilis* MtnK together and with the Pfam 016533 consensus of choline kinases (CHLK_CONS) obtained after Blast search of the WWWDDL at the NCBI. Codes as in Figure 1. BACSU: *B. subtilis*; BACST: *B. stearothermophilus*; BACA: *B. anthracis*; TREDE: *Treponema denticola*; KLEPN: *Klebsiella pneumoniae*; MESLO: *Mesorhizobium loti*; SINME: *Sinorhizobium meliloti*; ARATH: *Arabidopsis thaliana*. The sequences were extracted from the databases presented in [14].

none of these steady-state growth conditions (different sulfur, carbon and nitrogen sources) was there any significant effect of MTR on the gene expression (Table 1).

Table 1: Expression of *mtnK::lacZ* transcriptional fusions.

Medium used ^a	Strain	β -galactosidase Activity (U mg ⁻¹ of protein)	
		No MTR added	MTR added
ED1 minimal medium	BFS1850 ^b	1550	1650
methionine	BFS1850	1275	1250
isethionate	BFS1850	1800	1700
coenzymeM	BFS1850	1400	1370
taurine [§]	BFS1850	5000	4600
proline [§]	BFS1850	2800	2600
xylose + ammonium [§]	BFS1850	6500	ND
xylose [§]	BFS1850	3400	ND
ED1 minimal medium	BSHP1850 ^c	1660	1700
methionine	BSHP1850	1350	1300

a. for the β -galactosidase activity assay the bacteria were grown in the ED minimal medium as standard conditions. When another one replaced one of these glutamine, glucose and MgSO₄ components, only that change is indicated (for concentrations see Materials and Methods). b. BFS1850 = *mtnK::lacZ* c. BSHP1850 = *speD::spc mtnK::lacZ* §. starvation conditions

This observation was substantiated in expression profiling experiments (transcriptome analysis, see Materials and Methods) where growth on MTR was compared to growth on methionine: interestingly, *mtnK* and *mtnS* were amongst the highly expressed genes, but no significant difference was found in their transcription level under both conditions (Table 2). *mtnS*, in particular, was expressed at a level comparable to that of proteins of the translation machinery. Because MTR is naturally present in the cell due to polyamine biosynthesis (see also above), we used as a further control a strain unable to perform SAM decarboxylation and thus unable to produce high levels of MTA. In this background (*speD::spc mtnK::lacZ*, strain BSHP1850), MTR also failed to induce the operon expression (Table 1).

In contrast, sulfur, nitrogen and carbon starvation all induced *mtnK* expression. The factors of induction observed were dependent on the nature of the starved metabolites and varied between 2 to 5-fold, corresponding to a quite high level of protein expression (Table 1). The highest induction values were observed with carbon starvation conditions (5 times), when xylose was used as carbon source combined with relatively poor nitrogen source – ammonium (ammonium itself is not responsi-

Table 2: Hybridization on DNA membranes of cDNA from transcripts expressed in cells grown with MTR or methionine as the sulfur source (normalized arbitrary units).

	MTR A	Met A	MTR B	Met B
<i>mtnS</i>	4.6	4.4	4.5	4.6
<i>mtnK</i>	1.90	1.13	1.24	1.37
<i>mtnU</i>	0.68	0.58	0.60	0.67
<i>mtnV</i>	0.95	0.59	0.61	0.83
<i>mtnW</i>	0.91	0.81	0.83	0.80
<i>mtnX</i>	2.55	2.65	2.86	1.70
<i>mtnY</i>	1.11	0.74	0.68	0.73
<i>mtnZ</i>	0.98	0.96	0.92	0.98
<i>rplJ</i>	4.9	5.6	5.6	5.1
<i>tufA</i>	5.2	5.3	5.0	5.8
<i>gyrA</i>	4.3	5.3	4.8	4.1
<i>polA</i>	0.75	0.78	0.84	0.84
<i>polC</i>	0.77	0.89	0.80	0.98
Blank	0.22	0.23	0.27	0.24

Two different DNA membranes were used (A and B). The transcription hybridization level is given for genes at the *mtn* locus and, for comparison, genes expressed at a high level in the same experiment (translation: *rplJ* and *tufA*, replication and recombination: *polA* and *polC*, and DNA supercoiling: *gyrA*)

ble for this induction, since in the medium containing glucose and ammonium no induction was observed, data not shown). During sulfur starvation conditions (taurine as sulfur source), induction of *mtnK* expression was about 3.5 times. The lowest induction level was observed during nitrogen limitation (proline as nitrogen source) and was only about 2-fold.

Discussion

The fate of by-products of most metabolic reactions is usually forgotten, and not much work has been devoted to their recycling. Because sulfur metabolism is energetically costly it was interesting to investigate the fate of MTA, the major by-product of polyamine metabolism [17]. Furthermore, because polyamines are ubiquitous, but involved in yet uncharacterized processes, any hint about their function might be rewarding in terms of understanding the cell processes as well as in inventing new drug targets. We have identified in *B. subtilis* the first step of MTA degradation, hydrolysis to MTR [3]. Because we also showed that MTR was an excellent sulfur source for *B. subtilis* (this is in line with the plant biotope of this organism, since plants produce important levels of MTA during ethylene synthesis, for example), it was necessary to identify the corresponding recycling pathway. In all organisms where the question has been asked, it has been found that the first step is phosphorylation of MTR to MTR-1-phosphate. We looked therefore for a ki-

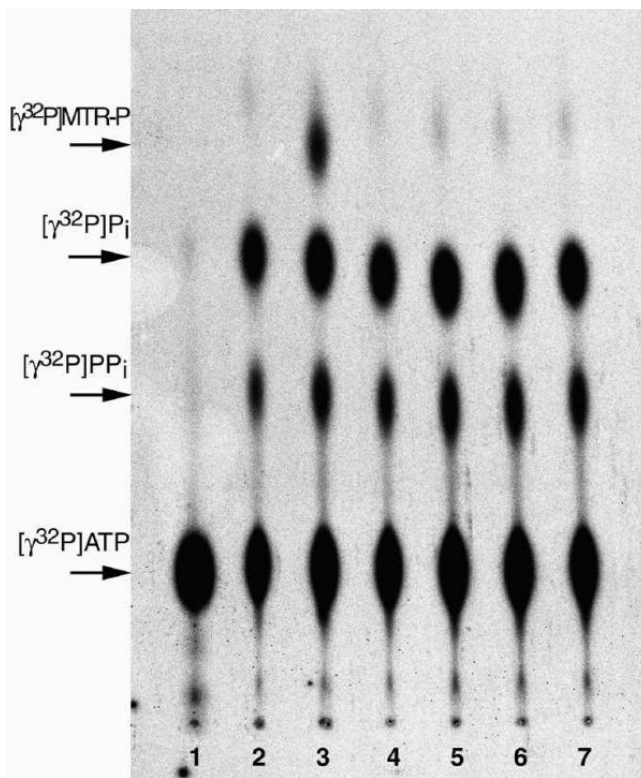


Figure 3

Autoradiograph showing the outcome of the MTR kinase assay. The assay was carried out for 90 min at 37°C (see Materials and Methods). Lane 1 corresponds to [^{32}P]ATP as standard; lanes 2, 4 and 6: no MTR was added for the reactions with cell-free extracts of wild type, BFS1850 and BFS1850 grown with IPTG, respectively; lanes 3, 5 and 7: reaction performed in the presence of MTR and cell-free extracts of wild type, BFS1850 and BFS1850 grown with IPTG, respectively.

nase that would fit the missing step. Gene *ykrT* was a possible candidate, since it codes for a protein of the choline/ethanolamine kinase family, while it belongs to a S-box regulated transcription unit [11]. Two types of genetic experiment substantiated this hypothesis: the *ykrT::lacZ* fusion constructed during the *B. subtilis* functional analysis program [18] failed to grow on MTR as a sulfur source, and more importantly, selecting for 3F-MTR resistance after transposon mutagenesis yielded an insertion in the leader mRNA of the *ykrTS* operon. Our study further demonstrated that both genes of this operon (which does not comprise the neighbour *mtnU* gene) are involved in MTR metabolism. We further showed biochemically that YkrT is indeed a MTR kinase, and named it accordingly MtnK. Using both expression profiling experiments and transcriptional fusions we demonstrated that both genes are expressed at a fairly high level. This contrasts with the observations published by Henkin and co-workers who found a much low-

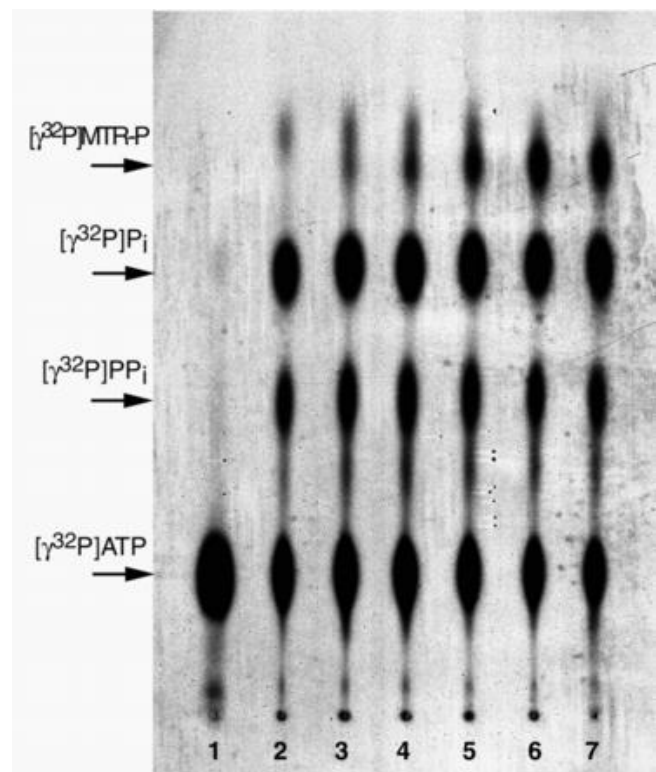


Figure 4

Autoradiograph of the MTR kinase assay with increasing concentrations of substrate. The assay was carried out for 90 min at 37°C with cell-free extracts of wild type (see Materials and Methods). Lane 1 corresponds to [^{32}P]ATP as standard; lanes 2: no MTR added; lanes 3, 4, 5, 6 and 7: reaction performed with increasing concentrations of MTR (20, 40, 80, 160 and 320 μM , respectively). Saturation is observed at 160 μM .

er expression of these genes [11]. Furthermore, we did not find significant differences in the expression factor of *mtnK* and in its repression factor by methionine in contrast to the data presented in the same Henkin *et al.* article. We could not find any straightforward explanation for this discrepancy. However, because starvation conditions appear to play an important role in *mtnK* expression it may be that subtle differences in growth conditions result in a drastic alteration of the expression pattern of the *mtnKmtnS* operon. Indeed, we have observed, using transcriptome experiments, that some gene expression is extremely sensitive to environmental conditions [19]. In addition, it should be remarked that our *lacZ* fusions have been constructed *in situ*, while they are located in a different strain at the distant SP β phage locus in the chromosome in Henkin *et al.* study [11]. Finally, our study shows no sulfur effect under steady-state conditions, while starvation for carbon, nitrogen or sulfur results in a strong enhancement of *mtnKS* synthesis. This may account for the difference in our experiments,

while showing that these genes belong to the starvation condition regulon.

That MtnK belongs to the choline kinase family Pfam 01633 is unexpected and interesting [20]. This is consistent with the involvement of a catalytic site binding the N-methyl or S-methyl group as important for recognition of the substrate, and not with a straightforward evolution from the kinases which phosphorylate ribose derivatives. This family is a member of a much wider Cluster of Orthologous Genes (COG0510) group, which belongs to a group of many other kinases, including carbohydrate antibiotic resistance gene (COG 3570) and serine/threonine protein kinases (COG 0661, COG 3178) [21]. Most conserved residues are charged residues, suggesting that they may be important in the catalytic activity of the enzyme. It would be of interest to explore further the phylogeny of these enzymes, basing alignments on gap and insertions rather than simply on similar or identical aminoacids [15,22].

Finally, the function of the *mtnS* gene remains elusive: while it seems to be necessary for the use of MTR as a sulfur source (in the context of the *B. subtilis* genes as they are grouped together) it does not seem to be necessary for the first step of MTR recycling. Further work is needed to delineate its function. MtnK is induced at a fairly high level under starvation conditions (with MtnS expressed at a very high level). This is consistent with adaptation of the cell to famine conditions, where byproducts need to be scavenged from the cytoplasm and from the environment, rather than lost. This may also be related to quorum-sensing signalling, which is known to occur under similar conditions. It would therefore be interesting to explore the relationship between the use of such by-products, cell density and quorum sensing.

Conclusion

MtnK performs the first step in MTR recycling. Consistent with the fact that MTR is a by-product of an anabolic pathway, MtnK expression is enhanced in starvation conditions. Isolation of 3F-MTR resistant mutants also yielded several other types of inserts failing to grow on MTR, thus demonstrating that 3F-MTR is phosphorylated and that downstream derivatives of this molecule are toxic to the cell. In particular, *ykrW* and *ykrY* insertion mutants were resistant to this toxic metabolite. These genes all belong to a neighbouring S-box regulated operon, and, despite the fact that they are quite dissimilar to the genes involved in *K. pneumoniae* MTR metabolism, this indicates that they are needed for recycling the sulfur moiety of this molecule, probably with similar chemical steps enacted by enzymes recruited from another group of proteins [23]. Work in progress is characterizing the corresponding activities and regulations.

Materials and Methods

Bacterial strains and plasmids, and growth media

E. coli and *B. subtilis* strains as well as plasmids used in this work are listed in Table 3. Despite the fact that there are no regulations yet in this domain in China, all experiments were performed in accordance with the European regulation requirements concerning the contained use of Genetically Modified Organisms of Group-I (French agreement N° 2735). *E. coli* and *B. subtilis* were grown in Luria-Bertani (LB) medium [24] and in ED minimal medium: K₂HPO₄, 8 mM; KH₂PO₄, 4,4 mM; glucose, 27 mM; Na₃-citrate, 0.3 mM; L-glutamine, 15 mM; L-tryptophan, 0.244 mM; ferric citrate, 33.5 μM; MgSO₄, 2 mM; MgCl₂, 0.61 mM; CaCl₂, 49.5 μM; FeCl₃, 49.9 μM; MnCl₂, 5.05 μM; ZnCl₂, 12.4 μM; CuCl₂, 2.52 μM; CoCl₂, 2.5 μM; Na₂MoO₄, 2.48 μM. For tests for growth with various sulfur sources, a sulfur-free medium was used. This medium was as described above but MgSO₄ was replaced by MgCl₂ at the same concentration (2 mM); the sulfur source tested were added at the followings concentrations: taurine, 5 mM; isethionate and coenzyme M, 2 mM; methionine, 1 mM; MTR, 0.2 mM. For nitrogen starvation, glutamine was replaced by proline (15 mM) and for carbon starvation, glucose was replaced by xylose (0.5%) and glutamine by ammonium (15 mM). For assaying growth on plates, either the MgSO₄ containing medium or the sulfur-free basal medium was used. In the latter case, 10 μl of the sulfur source under investigation was applied onto paper discs (MTR, 200 mM stock solution and methionine, 100 mM stock solution) deposited at the center of the plate, after bacteria had been uniformly spread at the surface of the plate, and growth was measured around the disk. When necessary IPTG was included at 1 mM concentration. LB and ED plates were prepared by addition of 17 g/liter Bacto agar or Agar Noble (Difco) respectively to the medium. When included, antibiotics were added to the following concentrations: ampicillin, 100 mg/liter; spectinomycin, 100 mg/liter; erythromycin plus lincomycin, 1 mg/liter and 25 mg/liter. Bacteria were grown at 37°C. The optical density (OD) of bacterial cultures was measured at 600 nm. MTR was prepared from MTA (Sigma, D5011) by acid hydrolysis as described by Schlenk [25]. 3-fluoromethylthiorybose (3F-MTR, 5-thio-5-S-trifluoromethyl-D-ribose) was synthesised accordingly to [26].

Transformation

Standard procedures were used to transform *E. coli* [27] and transformants were selected on LB plates containing ampicillin or ampicillin plus spectinomycin. *B. subtilis* cells were transformed with plasmid DNA following the two-step protocol described previously [28]. Transformants were selected on LB plates containing erythromycin plus lincomycin or spectinomycin or spectinomycin plus erythromycin plus lincomycin.

Table 3: 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⁻ F' [proAB⁺ lacI^q lacZ Δ M15 Tn10(tet^R)]</i>	Laboratory collection
<i>Bacillus subtilis</i>		
168	<i>trpC2</i>	[36]
BSIP7004	<i>trpC2 speD::spc</i>	[2]
BFS1850	<i>trpC2mtnK::lacZ</i>	This work ^a
BFS1851	<i>trpC2mtnU::lacZ</i>	This work ^a
BSHPI850	<i>trpC2speD::spc mtnT::lacZ</i>	This work
BSHP7010	<i>trpC2 mtnS::spc</i>	This work
BSHP7035	<i>trpC2pmtnK::Tn10</i>	This work
Plasmids		
pUC19	cloning vector, Amp ^R	[37]
pIC333	mini-Tn10 delivery vector, Spc ^R , Ery ^R	[32]
pMutin4mcs	cloning vector, Erm ^R	[31]
pDU1850	pMutin4mcs:: <i>mtnK</i>	This work
pDU1851	pMutin4mcs:: <i>mtnU</i>	This work
pHPP7010	pUC19 <i>mtnS::spc</i> ^b	This work

a. This strain has been constructed during the frame of the EC project for the functional characterization of the genome of *B. subtilis* in Europe

b. *spc* is the spectinomycin resistance gene from *Staphylococcus aureus*

Molecular genetics procedures

Plasmid DNA was prepared from *E. coli* by standard procedures [27]. *B. subtilis* chromosomal DNA was purified as described by Saunders [29]. 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). Amplified fragments were purified by QIAquick PCR Purification Kit (Qiagen). DNA fragments were purified from a gel using Spin-X columns from Corning Costar by subsequent centrifugation and precipitation.

To construct the *ykrS* deletion strain, a *Sma*I restricted spectinomycin resistance cassette [30] was used. Two DNA fragments, one upstream from the *ykrS* gene (nucleotides -490 to +35 relative to the translational start point of *ykrS*) and the second one downstream from the *ykrS* gene (nucleotides -9 to +452 relative to the *ykrS* stop codon) were amplified by PCR using primers introducing, for the first one, a *Bam*HI cloning site at the 5' end and a *Sma*I cloning site at the 3' end of the fragment, and for the second one, a *Sma*I cloning site at the 5' end and an *Eco*RI site at the 3' end of the fragment. PCR

products and the spectinomycin cassette were ligated and inserted into the *Bam*HI and *Eco*RI sites of pUC19 (Roche) producing plasmid pHPP7010. Prior to transformation, this plasmid was linearised at its unique *Sca*I site. Complete deletion of the gene was obtained by a double cross-over event, giving strain BSHP7010.

The DNA downstream from the *mtnK* gene (nucleotides +295 to +604 relative to the translation start point) was amplified by PCR using primers introducing an *Eco*RI cloning site at the 5' end and a *Bam*HI cloning site at the 3' end of the fragment, then inserted into the *Eco*RI and *Bam*HI sites of plasmid pMutin4mcs [31] producing plasmid pDU1850. Plasmids disrupting the *mtnU* gene was obtained by PCR amplification of downstream regions of *mtnU* gene (+105 to +346) as described for pDU1850, producing the plasmids pDU1851. Both plasmids were introduced into the chromosome by a single cross-over event, giving strains BFS1850 and BFS1851, respectively.

Transposon mutagenesis

Transposon bank was constructed by introduction of mini-Tn10 delivery vector pIC333 [32] into *B. subtilis* 168 strain as described previously [33]. Several thousands independent clones were pooled together and 5 samples of chromosomal DNA were prepared for further use. To obtain 3F-MTR resistant clones, *B. subtilis* 168 was transformed with chromosomal DNA containing previously prepared transposon banks and clones were selected on LB plates containing spectinomycin. Then, using velvets replicas, clones were transferred onto minimal medium plates containing 3F-MTR at 100 μM concentration and allowed to grow for 24 hrs. The single transposon insertion event was confirmed by back-cross in 168 strain and check for 3F-MTR resistance. To determine the location of the transposon insertion, chromosomal DNA was prepared, followed by subsequent digestion with *Hind*III, self ligation in *E. coli* XL1-Blue strain and plasmid sequencing. The primers used for sequencing of transposon insertions were the followings: Tn10 left: 5'GGCCGATTCATTAATGCAGGG3' and Tn10 right: 5'CGATATTCACGGTTTACCCAC3'.

RNA isolation and transcriptome analysis

Total RNA was obtained from cells growing on ED1 of 0.5 using "High Pure RNA Isolation Kit" from Roche (for RT-PCR minimal medium to an OD₆₀₀ experiment) or as described in [19] for transcriptome analysis. RT-PCR experiments were performed using RT-PCR System (Promega) as specified by the manufacturer. For cDNA synthesis in macro-array study, CDS-specific primers (cDNA Labelling primers – optimized for *B. subtilis*, Sigma-GenoSys Biotechnologies, Inc.) and two quantities of total RNA (1 and 10 μg) were used. Hybridization probes

were synthesized as described in [19]. Approximately 60–75% incorporation of labeled nucleotides was achieved in these conditions. Panorama™ *B. subtilis* gene arrays (Sigma-GenoSys Biotechnologies, Inc.) were used and the transcript levels corresponding to the genes analyzed in the present study were kept for further study. Since in the present study we did not require a thorough statistical analysis of the transcriptome data (performed in [19]), each experiment was averaged and the average was used as a standard for the analysis, since there was no obvious difference between the MTR and methionine growth conditions. Data presented here are normalized for each of these conditions.

Preparation of cell-free extracts

B. subtilis was grown to middle-exponential growth phase (~0.7–0.8). The organisms were harvested by centrifugation (14,000 rpm, 4°C, 3 min), and the (OD₆₀₀ cell pellets were resuspended in a solution containing 150 mM glycine (pH 9) and 1 mM β-mercaptoethanol (β-ME). The bacterial cells were disrupted by sonication (4 min, maximum amplitude) and cellular debris was removed by centrifugation (14,000 rpm, 4°C, 10 min).

Enzyme assays

B. subtilis cells containing *lacZ* fusions were assayed for β-galactosidase activity as described previously [34]. Specific activity was expressed in Units per mg protein. The Unit used is equivalent to 0.28 nmols min⁻¹ at 28°C. Protein concentration was determined by Bradford's method using a protein assay Kit (Bio-Rad Laboratories). At least two independent cultures were monitored.

MTR kinase was assayed as described by [16] with minor modifications (glycerol was present as a stabilizer in the first experiments, while it was absent in the final ones because we found that MtnK phosphorylated this molecule). Briefly, the reaction mixture of 100 μl contained 150 mM glycine (pH 9), 1 mM MgCl₂, 1 mM β-ME, 40 μM MTR, 0.15 μg/ml crude extract (final) and 1 mM [γ-³²P] ATP. [γ-³²P] ATP (10 Ci/mmol, Dupont-New England Nuclear) was diluted with nonradioactive ATP to yield a 10 mM stock solution. Specific activity of the stock ATP mixture was 20 μCi/μmol (0.2 μCi/μl). The reactions were carried out at 37°C for 90 min. After this period, reactions were terminated by sitting tubes on ice. [γ-³²P] MTR-P was separated from [γ³²P] ATP, ³²PP_i and ³²P_i on PEI-Cellulose F plates Merck as described by [35]. 1 μl samples were loaded on the plate and separated with 1 M LiCl. Samples were allowed to resolve until the solvent front was about 1 cm below the edge of plate (approximately 40 min). The plate was dried, and the radioactivity was detected by autoradiography using Biomax-MR Kodak film.

List of Abbreviations

bp: base pairs; β-ME, β-mercaptoethanol; CDS: coding sequence; IPTG: isopropyl β-D-thiogalactopyranoside; MTA: methylthioadenosine; MTR: methylthioribose; 3F-MTR: trifluoromethylthioribose; nt, nucleotides

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