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Research article

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Bacterial variations on the methionine salvage pathway

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Abstract

Background: The thiomethyl group of S-adenosylmethionine is often recycled as methionine from methylthioadenosine. The corresponding pathway has been unravelled in *Bacillus subtilis*. However methylthioadenosine is subjected to alternative degradative pathways depending on the organism.

Results: This work uses genome in silico analysis to propose methionine salvage pathways for *Klebsiella pneumoniae*, *Leptospira interrogans*, *Thermoanaerobacter tengcongensis* and *Xylella fastidiosa*. Experiments performed with mutants of *B. subtilis* and *Pseudomonas aeruginosa* substantiate the hypotheses proposed. The enzymes that catalyze the reactions are recruited from a variety of origins. The first, ubiquitous, enzyme of the pathway, MtnA (methylthioribose-1-phosphate isomerase), belongs to a family of proteins related to eukaryotic initiation factor 2B alpha. *mtnB* codes for a methylthioribulose-1-phosphate dehydratase. Two reactions follow, that of an enolase and that of a phosphatase. While in *B. subtilis* this is performed by two distinct polypeptides, in the other organisms analyzed here an enolase-phosphatase yields 1,2-dihydroxy-3-keto-5-methylthiopentene. In the presence of dioxygen an aci-reductone dioxygenase yields the immediate precursor of methionine, ketomethylthiobutyrate. Under some conditions this enzyme produces carbon monoxide in *B. subtilis*, suggesting a route for a new gaseous mediator in bacteria. Ketomethylthiobutyrate is finally transaminated by an aminotransferase that exists usually as a broad specificity enzyme (often able to transaminate aromatic aminoacid keto-acid precursors or histidinol-phosphate).

Conclusion: A functional methionine salvage pathway was experimentally demonstrated, for the first time, in *P. aeruginosa*. Apparently, methionine salvage pathways are frequent in Bacteria (and in Eukarya), with recruitment of different polypeptides to perform the needed reactions (an ancestor of a translation initiation factor and RuBisCO, as an enolase, in some Firmicutes). Many are highly dependent on the presence of oxygen, suggesting that the ecological niche may play an important role for the existence and/or metabolic steps of the pathway, even in phylogenetically related bacteria. Further work is needed to uncover the corresponding steps when dioxygen is scarce or absent (this is important to explore the presence of the pathway in Archaea). The thermophile *T. tengcongensis*, that thrives in the absence of oxygen, appears to possess the pathway. It will be an interesting link to uncover the missing reactions in anaerobic environments.

Background

Availability of a huge number of genome sequences (1004 ongoing or completed genome sequencing programmes are listed at <http://wit.integratedgenomics.com/GOLD/> on February 17th, 2004) now allows scientists to explore new avenues in the deciphering of metabolic pathways. While sulfur is ubiquitously used in life, it is a highly reactive atom, hence very sensitive to the oxido-reduction potential of its local environment. It is also very sensitive to dioxygen and reactive oxygen species. As a consequence, sparing processes that enable the cell to keep a sulfur atom in its final chemical and electronic environment (inside well characterized groups of atoms) often exists in cells [1]. A case in point is the fate of the ubiquitous S-adenosylmethionine molecule (AdoMet), which can donate each one of its sulfur connected groups: methyl-, aminobutyryl- (usually, after decarboxylation as aminopropyl-), and adenosyl- (ribosyl-). A vast number of AdoMet mediated methylation events continuously happen in most, if not all organisms. Methylases are present at the level of DNA metabolism, RNA metabolism and protein post-translational modifications [2-4]. They are also involved in formation of a variety of small metabolites (essential coenzymes or prosthetic groups in particular [5,6]). This group transfer results in the synthesis of S-adenosyl homocysteine (AdoHcy). The latter molecule has to be recycled or further metabolized, either after excision of the adenine moiety by hydrolysis or phosphorolysis, or by direct hydrolysis into adenosine and homocysteine. When aminopropyl- is transferred, methylthioadenosine (MTA) is a by-product. It is produced during synthesis of spermidine and spermine, and also of N-acylhomoserine lactone synthases in Gram-negative bacteria [7] and 1-aminocyclopropane-1-carboxylate (ACC) synthase in plants (and possibly in bacteria producing ethylene). Furthermore, other still unknown reactions might exist, transferring the aminopropyl-group to yet unrecognized acceptors (most likely to be nitrogen atoms). Recycling the sulfur-containing compounds S-adenosylhomocysteine and MTA is a challenge for all cells. MTA may regenerate methionine in the process. The biochemical details of the MTA to methionine pathway remains unknown in most organisms. This prompted us to make a thorough analysis of available genome sequences to try and identify some of the metabolic pathways involved in MTA recycling through genomic and functional comparisons with our recent findings [8]. The present work aims at combining comparative genomics and biochemical or genetic data to propose a list of identified and putative genes associated to the corresponding functions. A self-consistent nomenclature will be proposed in parallel, so that homologous functions in various organisms could tentatively be identified from their genome sequences.

Results

MTA can be exported from the cell, or recycled, beginning with two main alternative pathways (Figure 1). The most energy sparing one starts with MTA phosphorolysis and yields methylthioribose-1-phosphate (MTR-1-P) [9], while the second one splits this first step into two steps: the first one being hydrolysis of MTA to methylthioribose (MTR) and adenine, followed by ATP-dependent phosphorylation of MTR by MTR kinase [10,11]. In this latter case, MTR can also be excreted from the cell, especially in organisms that are not challenged by a high level of dioxygen (this happens in facultative anaerobes living in rich media, such as *Escherichia coli* [12]).

MTA phosphorylase

The gene coding for MTA phosphorylase (named *mtnP*, see below) has been identified in many organisms, in all three kingdoms: Archaea, Bacteria and Eukarya. The activity of the enzyme has long been studied as a target to fight some kinds of tumor cells [13]. A PROSITE signature, PDOC00946, [GST]-x-G-[LIVM]-G-x-[PA]-S-x-[GSTA]-I-x(3)-E-L, is common to three classes of nucleoside phosphorylases, purine nucleosidases, uridine nucleosidases and MTA phosphorylase. A second signature, PDOC00954 [LIV]-x(3)-G-x(2)-H-x-[LIVMFY]-x(4)-[LIVMF]-x(3)-[ATV]-x(1,2)-[LIVM]-x-[ATV]-x(4)-[GN]-x(3,4)-[LIVMF](2)-x(2)-[STN]-[SA]-x-G-[GS]-[LIVM] labels some other phosphorylases (including MTA phosphorylase), from Eukarya in particular.

This family can be narrowed into three conserved domains families: COG0005, purine nucleoside phosphorylase, pfam00896, phosphorylase family 2, KOG3985, MTA phosphorylase. Interestingly, these purine nucleoside phosphorylase families share most of their sequence in amino acid residues, except for the very first residues, that appear to be made of a particular sequence in the case of MTA phosphorylase. In contrast to other purine nucleoside phosphorylases, it appears likely that the amino-terminal end of MTA phosphorylase starts very early upstream of the G [ST]G sequence that is conserved in purine nucleoside phosphorylases: the exception we find to the rule is the sequence present in *Synechocystis* PCC6803, but a methionine codon is placed just at the expected beginning of the protein, while translation of the upstream region is not confirmed by biochemical analysis (and it is notorious that start codons are difficult to identify in general and in cyanobacteria in particular). Immediately upstream of this first consensus sequence, a G amino acid residue is found in all known and putative MTA phosphorylases, as well as other features of the region displayed in Figure 2. It was therefore important to explore whether in *Pseudomonas aeruginosa* gene PA-3004 (*mtnP*), which has this signature, codes for MTA phosphorylase. The presence of this gene is not ubiq-

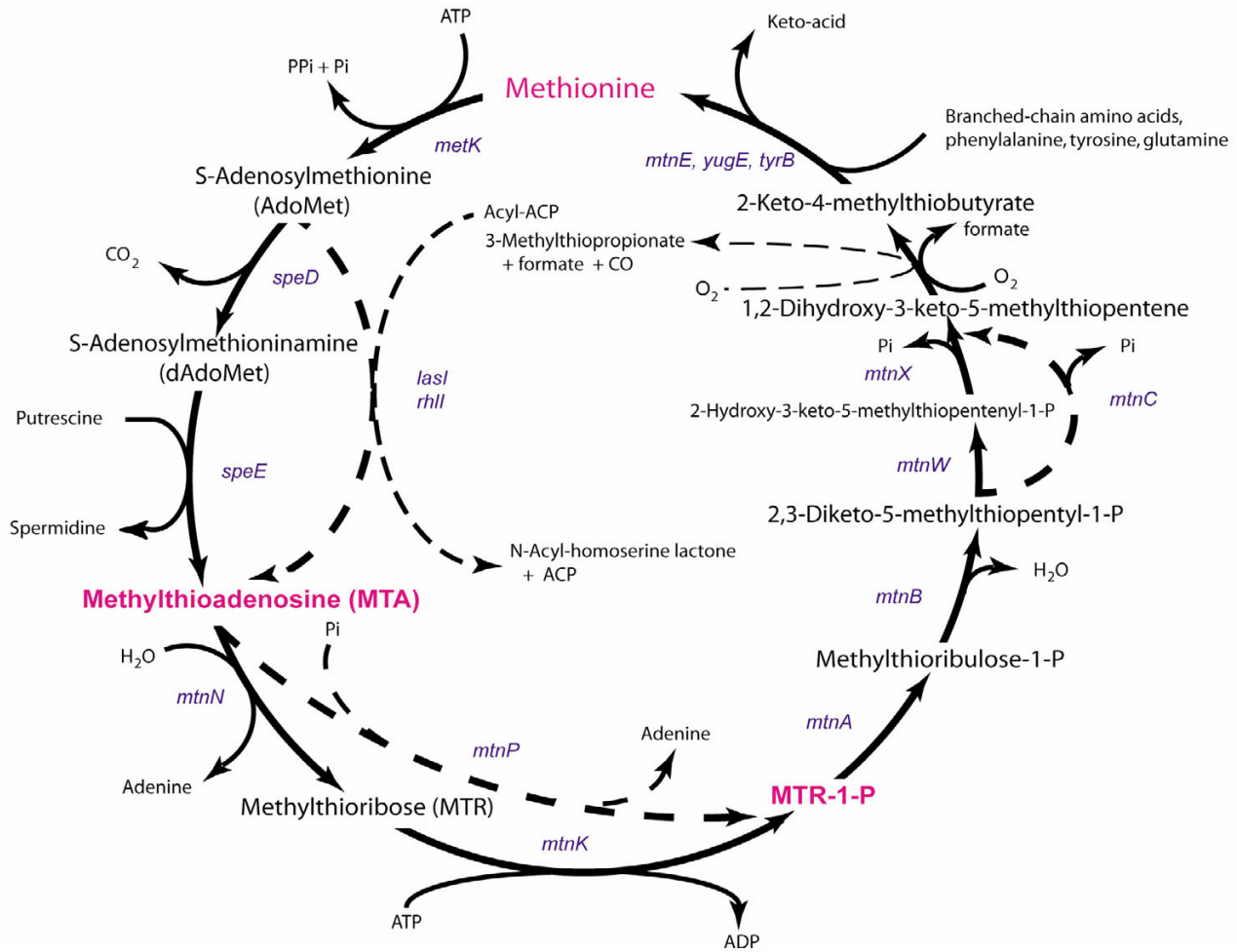


Figure 1
The methionine salvage pathway. As a reference, the pathway is shown in *B. subtilis* (solid lines). Variants of the pathway are shown in broken lines.

uitous in gamma-proteobacteria, since this gene seems to be absent from *Klebsiella pneumoniae* (the sequence of the complete genome is not publicly available, sequences of the ongoing genome project are available at <http://genome.wustl.edu/blast/client.pl>).

The *P. aeruginosa* methionine-auxotroph PAO503, which carries a mutation in the *metZ* gene encoding *O*-succinyl-homoserine sulfhydrylase [14], grew well on minimal medium E when supplemented with either methionine, homocysteine or MTA. By contrast, a mutant derived from PAO503 by gene replacement, PAO6422 (*metZ mtnP*), did not grow with MTA, but still responded to methionine and homocysteine (Table 1). These results demonstrate that *P. aeruginosa* has an MTA recycling pathway and that

MTA phosphorylase has an essential function in this pathway.

An alignment of MTA phosphorylase and purine phosphorylase is provided in Figure 2, with sequences from *Thermoanaerobacter tengcongensis* (Firmicutes [15]) and *P. aeruginosa* (gamma-proteobacteria), as well as from an organism that is usually difficult to classify in terms of proteins, *Leptospira interrogans*. In addition to the position of the beginning of the protein (shorter in MTA phosphorylase) residues that are discriminating between general scavenging purine phosphorylase and MTA phosphorylase are indicated in Figure 2, around the common motif [FIMLV]-[IV]-X-G-[ST]-G-X₁₆₋₂₂-P. Noteworthy features are the presence of conserved residues in MTA phosphor-

MTA phosphorylase

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PSEAE 1      MSVY AIIGGTGLTQLEGL TLS ES-----L- PIETTPYG APSA-PLQRGR YAG---RE VLFL 49
THETE 1      MEK AIIGGTGVYDVADK --- VV-----QK KVETTKYG TVEVDVVTVVEG ED----- IVFL 45
HOMSA 9      AVKI GIIGGTGLDDPEIL EGR TE-----K- YVDTPFG KPSDALILG-K IKN---VD CVLL 58
CAEEL 1      MVKV GIIGSGLEDPNIL LDP VT-----V- AVDTPYG KPSDDVVEG-T ING---VE CVLL 50
SCHPO 7      PILL GVIGSGFYDLPGF DIV ES-----V- NPITTPWG YPASPISIART TSG---FL IAFL 57
SYNEC (35)  MVNAPI GIIGSGLYQMEAL KNV EE-----V- TLDTPFG APSDSFIVG-E LAG---VR VAFI 84
MYCTU 5      GRML GVIGSGFYTFPGS DTR TV----- NSDTPYG QPSAPITIG-- TIGV--HD VAFI 53
METJA 1      MI GIIGGTGIAEILKG DKE E----- IINTTKYG KA-RVIIDK-- EN-----E VVLL 42
METTH 1      MI GIIGGTGIYEMAEY GRL ER-----RG SLITTPYG KTPEISVFK-- LHG---RR VAFI 48
LEPIN 1      MSYNVRA AIIGGTGLYSLEGM ELI EE-----I- FPDTPWG KPSDKIKIG-K YKG---KL IAFL 53
XYLFA 1      MQTIAL AVIGGTGVYTLSQL DDV QV-----Y- EVETLYG RPSGPIRVG-M LFG---QR VAFF 52
    
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Purine phosphorylase

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MYCTU 27     EHDV AVVLGSGWLPAAVA LGS PTT---VLP QAELPGF VPPTAAGHAGE LLSVPIGA HRVL 83
ESCCO 25     TPRV AFILGSGLALADQ IEN AVA---IS YEKLPGF PVSTVHGAGE LVLGHLQ VPVV 80
SACCE 37     PPRT LIICGSGLGGISTK LSR DNPPPVTVP YQDIPGF KKSTVPGHSGT LMFGSMNG SPVV 96
BACSU 19     SPKI GLILGSGLGLADE IEN PVK----LK YEDIPEF PVSTVEGHAGQ LVLGTLEG VSVI 74
GEOST 20     SPQI GLILGSGLGLADE IEQ AIK----IP YSDIPNF PVSTVEGHAGQ LVYGQLEG ATVV 75
BOSTA 24     RPQV AVICGSGLGLVKNK LTQ AQT---FD YSEIPNF PESTVPGHAGR LVFGILNG RACV 79
    
```

Figure 2

Comparison of purine and MTA phosphorylases. The beginning of MTA phosphorylases is aligned with that of purine phosphorylases extracted from phylogenetically widely diverse organisms: PSEAE *Pseudomonas aeruginosa*; THETE *Thermoanaerobacter tengcongensis*; HOMSA *Homo sapiens*; CAEEL *Caenorhabditis elegans*; SCHPO *Schizosaccharomyces pombe*; SYNEC *Synechocystis* PCC 6803; MYCTU *Mycobacterium tuberculosis*; METJA *Methanococcus jannaschii*; METTH *Methanobacterium thermoautotrophicum*; LEPIN *Leptospira interrogans*; XYLFA *Xylella fastidiosa*; ESCHO *Escherichia coli*; SACCE *Saccharomyces cerevisiae*; BACSU *Bacillus subtilis*; GEOST *Geobacillus stearothermophilus*; BOSTA *Bos taurus*. Conserved residues in both classes of enzymes are emphasized [54]. In red are shown conserved residues that are specific to one class, while conservative replacements are indicated in blue. The three residues in green indicate a region that suffered an insertion/deletion event in the course of evolution that separated the two classes. The position of the first residue of the alignment shown is displayed in blue. Note that for *Synechocystis* the published sequence starts earlier, but that the residue indicated is likely to be the real start of the protein.

Table 1: Growth of *Pseudomonas aeruginosa* mutants on various sulfur sources

Strain	MTA	Methionine	Homocysteine
PAO503 metZ901I	+	+	+
PAO6412 metZ901I mtnB::Ω-Sp/Sm	-	+	+
PAO6422 metZ901I mtnP::Ω-Sp/Sm	-	+	+ ^a

^a weaker growth as compared to the other constructs.

ylase, that differ from those at the same position in the other phosphorylases: G upstream of the highly conserved G-[ST]-G motif and a T is present upstream of the partially conserved P residue, with a downstream aromatic residue followed by a G in MTA phosphorylase (respectively L or I and F in other phosphorylases). The product of the reaction is MTR-1-P, which undergoes further processing as detailed below.

MTA hydrolysis and methylthioribose phosphorylation

AdoMet-dependent methylases produce AdoHcy, while aminopropyl-group transfer produces MTA. It was once thought that the metabolism of AdoHcy was split in various organisms into two alternative pathways [16]: the molecule was recycled either by AdoHcy hydrolase that generates homocysteine and adenosine, or by the sequential action of AdoHcy nucleosidase that generates adenine and S-ribosylhomocysteine, followed by a S-ribosylhomocysteine-cleavage enzyme. The hydrolase has been now found in many organisms, such as *Pseudomonas* sp., *Homo sapiens*, *L. interrogans*, *Aquifex* sp. and *Thermotoga maritima* but it is not present in *E. coli* nor in *Bacillus subtilis*, at least with a related sequence [17].

In *E. coli* the nucleosidase (coded by gene *pfs*) has in fact a lower specificity since it hydrolyses both AdoHcy and MTA [18]. In *B. subtilis* it has been experimentally demonstrated that the gene initially termed *yrU* has this activity [19]. The counterpart of this enzyme exists in *K. pneumoniae* (Figure 3). In many bacteria (including *E. coli*) the fate of the MTR thus produced is not known, but the molecule is found to accumulate in the external medium, suggesting the presence of an export system [12]. In *B. subtilis* experiments using radioactive labelling have shown that a kinase exists that transforms MTR into MTR-1-P (Figure 1). It is encoded by a gene belonging to a S-box riboswitch-controlled operon [20,21], *yrT* (renamed here *mtnK*, see below) [22]. In contrast with the situation in *E. coli*, a counterpart of this gene exists in *K. pneumoniae* (Figure 3), consistent with the existence of a methionine salvage pathway in the latter. Interestingly, this enzyme that has been crystallized recently [23] does not belong to the ribo- or deoxyribokinase family, but, rather, to the choline/ethanolamine kinase family [24].

The elusive downstream section of the methionine salvage pathway

It has long been known that MTA, once converted to adenine and MTR-1-P was able to regenerate methionine. This was found in organisms as distant as *H. sapiens* or *K. pneumoniae*. The overall reaction scheme has been unraveled by Abeles and coworkers in *K. pneumoniae* [25]. However, the actual enzymes or genes involved for each step have not been uncovered in totality. A gene inactivation study in *B. subtilis* revealed the involvement of several

genes [26], but the exact nature of the enzymes needed for each step in this organism was only discovered when appropriate biochemical experiments were set up [8]. Briefly, the pathway in *B. subtilis* begins as follows: MTR-1-P is converted into MTRu-1-P by the isomerase encoded by gene *yrS* (now *mtnA*, see new proposed nomenclature below) [8,22]. Remarkably, it has been observed that this gene, expressed at a level similar to that of *yrT* (*mtnK*) [22], codes for a protein with a sequence annotated as similar to that of eukaryotic initiation factor eIF-2B (alpha subunit). Figure 4 shows a high degree of conserved residues in the corresponding alignment with genes from Firmicutes, gamma-proteobacteria, plants and humans. Interestingly this sequence is similar, especially in its carboxy-terminal end (see the *Mus musculus* sequence), with eIF-2B-alpha, but, as can be seen in the Figure, this similarity, although unambiguous, should perhaps not have been emphasized in genome annotations.

The next step is dehydration of MTRu-1-P by MTRu-1-P dehydratase encoded by gene *yrY* (now *mtnB*), a member of operon *yrWXYZ*, under the control of an S-box riboswitch [20,21]. The product of the reaction is the diketone 2,3-diketo-5-methylthiopentyl-1-phosphate. A counterpart of this gene can be found in many organisms (Figure 5), where it is usually associated to other genes involved in the pathway. This is the case, for example in *K. pneumoniae*. In *P. aeruginosa*, the operon *aroC*, PA-1682, PA-1683, PA-1684, *mas* <http://bioinfo.hku.hk/AeruList> encodes proteins similar to chorismate synthase, a metabolite transporter, MTRu-1-P dehydratase, 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase and enolase-phosphatase, respectively. Indeed, the methionine-auxotroph (*metZ* [14]) *P. aeruginosa* strain PAO503, which grew well on minimal medium E when supplemented with either methionine, homocysteine or MTA, lost the ability to use MTA when the *mtnB* gene (PA-1683) was inactivated by gene replacement in strain PAO6412 (*metZ mtnB*) (Table 1). Thus, in addition to the MTA phosphorylase, *P. aeruginosa* has an MTRu-1-P dehydratase which has an essential function in the methionine salvage pathway (Table 1). The *MtnB* sequence is a member of a large cluster of proteins that form class II aldolases (COG235, Pfam0096). Among these enzymes one finds L-ribulose-5-phosphate-4-epimerase, suggesting that recognition of the ribulose backbone is common to a variety of enzymes of the class. The N-terminal domain of these proteins form class KOG2631, which appears to overlap exactly with the dehydratase.

The dehydratase product is further metabolized by 2,3-diketo-5-methylthiopentyl-1-phosphate enolase, encoded in *B. subtilis* by gene *yrW* (now *mtnW*). Interestingly, this enzyme is highly similar to RuBisCO [8,26,27]. It produces 2-hydroxy-3-keto-5-methylthiopentyl-1-

MtnN : Methylthioadenosine nucleosidase

BACSU	1	MRLAVIGAMEEVTILRNKLENAKTETIAHCEFTTGEYEGTEVILLKSGIGKVNAAI	60
ESCCO	1	MKIGIIGAMEEVTLLRDKIENRQTISLGGCEIYTGQNGTEVALLKSGIGKVAALGAT	60
KLEPN	1	MKIGIIGAMEEVTLLRDKIENRQITITIGGSEIYTGQLHGVDVALLKSGIGKVAAMGAT	60
BACSU	61	LLLDRYKPDYVINTGSAGGFHHTLNVGDVVISDVRHHDVDTAFDYEYQGVPLPAAYA	120
ESCCO	61	LLLEHCKPDVIINTGSAGGLAPTLKVGDIIVVSEARYHDADVTAFGYEYQGLPCPAGFK	120
KLEPN	61	LLLERCQPDVIINTGSAGGLAS TLKVGDIIVVSEARYHDADVTAFGYEYQGLPCPAGFK	120
BACSU	121	ADEKLISITEEAVSELDGIQVAKGTIATGDSFMNDPKRVEEVRARFSDLYAVEMEA	180
ESCCO	121	ADDKLIAAAEEACIAELN-LNAVRGLIVSGDAFINGSVGLAKIRHNFQAI	179
KLEPN	121	ADEKLVAAEESCICALD-LNAVRGLIVSGDAFINGSVGLAKIRHNFQAI	179
BACSU	181	QVCHQFKTPFVVI RALSDIAGKESHVSFDQFLEQAAVHSTELVLKVIKRI	230
ESCCO	180	HVCHNFVFPFVVVRAISDVADQQSHLSFDEFLAVAAKQSSLMVESLVQKLAHG	232
KLEPN	180	HVCHNFKVPFVVVRAISDVADQQSHLSFEEFLAVAAARQSTLMVENLVQNL	229

MtnK : Methylthioribose kinase

BACSU		MGVTKTPLYETLNESSAVALAVKL-GLFPSKSTLTCQEI	IGDGNLNYVPHIYDQEHDRALI	59	
KLEPN		MSQYHTFTAHD	AVAYAQQFAGIDNPSELVSAQEVGDGNLNLVFKVFD	RQGVSRAI 55	
XYLFA		MHPDNALKQPHLEALHQSSATALLVLI	NLSRRNSATATLRAAGNTPSAL	TMPC* 55	
BACSU	60	IKQAVPYAKVVGESWPLTIDRARIESSALIRQGEHVPHLVP	RVFYSDETEMAVTVMEDLSH	119	
KLEPN	56	VKQALPYVRCVGESWPLTLDRARLEAQT	LVAHYQHSQHTV	KIHHFDPELAVMVMEDLSD 115	
BACSU	120	LKIARKGLIEGENYPHLSQHIGEF	LKTLFYSDDYALEPKVKKQLVKQFTNPEL	CDITER 179	
KLEPN	116	HRIRGELIANVYYPQAA	RQLGDYLAQVLFHTSDFYLHPHEKKAQVAQFINP	AMCEITED 175	
BACSU	180	LVFTDPFFDHD	TNDFEEELRPFVEKLWNNDSVKIEAAK	LKKSFLTSAETLIHGD	LHTGSI 239
KLEPN	176	LPFNDPYQIHERNNYPAELEADVAAL	RDDAQLKLAVAALKHRFFAHAEALLHGD	IHSGSI 235	
BACSU	240	FASEHETKVIDPEFAYGPIGFDV	QGFIANLFLNALS-----RDGAD-REPLYEHV	NQ 291	
KLEPN	236	FVAEGSLKAIDAEFGYFGPIGFD	IGTAIGNLLNVCGLPGQLGIRDAAAAREQRL	NDIHQ 295	
BACSU	292	VWETFEE	TFSEAWQKDSLQVYANIDGYLTD	TLSHIFEEAIGFAGCELIRRTI	GLAHVADL 351
KLEPN	296	LWTTFAERFQALAAEKTRDAALAYP	GYASAFLLKQVWADAVGFCGSELIRRSV	GLSHVADI 355	
BACSU	352	DTIVPFDKIRGRKRLALETG	TAFIEKRSEFKTITDVI	ELFKLLVKE 398	
KLEPN	356	DTIQDDAMRHECLRHAITLGRALI	VLAERIDSVDELLARVRQYS	399	

Figure 3

The MTA nucleosidase / MTR kinase pathway in *B. subtilis* and *K. aeruginosa*. When the methionine salvage pathway exists, while the organism does not code for a MTA phosphorylase, the combination of MTA nucleosidase and MTR kinase produce MTR-I-P from MTA (Figure 1). *B. subtilis* (BACSU) and *K. pneumoniae* (KLEPN) possess this pathway, while *E. coli* (ESCCO) has only the nucleosidase. Conservative replacements are shown in blue following Dayhoff's rule [54]. The short protein that may be a truncated MtnK in *X. fastidiosa* is shown for comparison (identities with the *B. subtilis* sequence in orange, and similarities in violet).



Figure 4

Alignment of methylthioribose-1-phosphate isomerases. Conserved residues are indicated in red, while conservative replacements are indicated in blue. Organisms are labelled as in Figure 2, with a few additional organisms: AQUAE *Aquifex aeolicus*; ARATH *Arabidopsis thaliana* and MUSMU *Mus musculus*. The sequence of mouse initiation factor eIF-2B (alpha subunit) is shown for comparison. The § symbol at the beginning and the end of the *K. aeruginosa* sequence corresponds to regions that have not been identified from the ongoing genome programme sequences.



Figure 5

Alignment of methylthioribulose-1-phosphate dehydratases. Important residues are indicated as in Figure 2. For *A. thaliana* (sequence from At5g53850) the N-terminal end of the protein is aligned. The § symbol at the beginning and the end of the *K. aeruginosa* sequence corresponds to regions that have not been identified from the ongoing genome program sequences.

phosphate. At this stage (outcome of the enolase reaction) 2-hydroxy-3-keto-5-methylthiopentenyl-1-phosphate phosphatase, encoded in *B. subtilis* by gene *ykrX* (*mtnX*), produces 1,2-dihydroxy-3-keto-5-methylthiopentene (Figure 1).

In other organisms, such as *K. pneumoniae*, the corresponding enzymes appear usually to consist of a single enolase-phosphatase, allowing the cell to bypass the last step. Interestingly, in *Arabidopsis thaliana*, the protein carrying the dehydratase (Figure 5) is a two-domain protein, the carboxy-terminal domain coding for the enolase-phosphatase (Figure 6). This further substantiates the proposed identification of the corresponding steps in the pathway. The enolase-phosphatase corresponds to the KOG family KOG2630. The product of the enolase-phosphatase reaction is then submitted to the action of a dioxygenase, as demonstrated in *K. pneumoniae* [28]. It has also been shown that dioxygen is required for the methionine salvage pathway in *B. subtilis* [8,26]. The sequence of the proteins is illustrated in Figure 7. The aci-reductone dioxygenase that creates 2-keto-4-methylthiobutyrate (KMTB) has counterparts in many Bacteria and Eukarya, including in mammals, belonging to family COG1791. It seems to be absent from Archaea. Interestingly Abeles and co-workers discovered that the enzyme has two activities, depending on its metal co-factor. When iron was involved, the enzyme produced KMTB, the immediate precursor of methionine, together with formate. In contrast, when nickel was involved, carbon monoxide was produced in addition to formate, together with 3-methylthiopropionate [29]. The enzyme has been crystallized [30]. In *B. subtilis*, using palladium salt reduction, we found that CO was likely to be produced when the cells were grown in the presence of MTR. A significant difference between the wild type and a *mtnD* mutant could be seen: the wild type makes more reducing gasses when MTR is the sulfur source (16.7 ppm/hour, Table 2). However, *B. subtilis* produced other reducing gasses as shown by the presence of a reddish-black hue in the precipitate, even in the mutant. To test for another possible source of reducing gasses we investigated reducing gas production in a *yflM* mutant. Its gene product, YfLM, is highly similar to an NO synthase. As shown in Table 2, an *yflM* mutant produced somewhat less reducing gasses. The amount produced in our conditions, about 6.7 ppm/hour less than the wild type in sulfate, and 10 ppm/hour less in MTR, is however not much higher than the uncertainty of the experiment (ca 5 ppm/hour). In this background, the effect of MTR was still visible and significant as compared to growth with sulfate as sulfur source (13.4 ppm/hour). However a significant level of palladium precipitation remains in these mutants. The background level found in the absence of bacteria was of the order of 12.5 ppm/hour. Other gasses such as methanethiol and hydrogen sulfide

are likely to contribute to the reducing power of the organism.

When MTA is recycled, the two last steps of the methionine salvage pathway appear to be common to all organisms that grow in the presence of dioxygen. The counterpart of the dioxygenase is not known in organisms that live under anaerobic growth conditions. KMTB is transaminated to methionine. There are many analogs of the final aminotransferase, however they can more or less complement one another (as is often the case with aminoacid aminotransferases, see e.g. [31]). In *B. subtilis*, putative aspartate aminotransferases, the *yugH*, *yufG*, *ykrV*, *aspB*, and *pataA* gene products, have been cloned, expressed, and characterized for methionine regeneration activity. In this family, only YkrV (encoded by *ykrV*, adjacent to the *ykrWXYZ* operon) was able to convert KMTB to methionine, and it catalyzed the reaction only when glutamine was used as amino donor [32]. This suggests a link with nitrogen metabolism, substantiating our previous observations [33]. However the bulk of the activity resulted from transaminase YbgE, using either branched chain aminoacids or phenylalanine or tyrosine as donors could also perform a similar reaction [32]. In *K. pneumoniae* it is the same enzyme that makes methionine from KMTB and tyrosine from its ketoacid precursor [34].

Nomenclature

We propose the following nomenclature, taking into consideration previous names of some of the genes, as well as previous usage of names for some other groups of genes, trying to avoid naming conflicts. The acronym "*mtn*" (for methylthionucleoside) will be used.

Because the early steps are variable, we shall use as a mnemonic the capital letter of the enzyme's name, following Demerec's rule. Thus, *mtnN* will code for MTA nucleosidase, *mtnK* will code for MTR kinase, *mtnP* will code for MTA phosphorylase.

Considering that the common part of the pathway starts with MTR-1-P, we shall name the following genes sequentially: *mtnA* will code for MTR-1-P isomerase (*ykrS* in *B. subtilis*) and *mtnB* will code for MTRu-1-P dehydratase (*ykrY*).

In *B. subtilis*, the pathway is probably unusual, with *mtnW* (*ykrW*) coding for the enolase and *mtnX* (*ykrX*) for the phosphatase creating 1,2-dihydroxy-3-keto-5-methylthiopentene. In other organisms, this step is performed by an enolase-phosphatase, encoded by gene *mtnC*, while *mtnD* (*ykrZ*) codes for the aci-reductone dioxygenase.

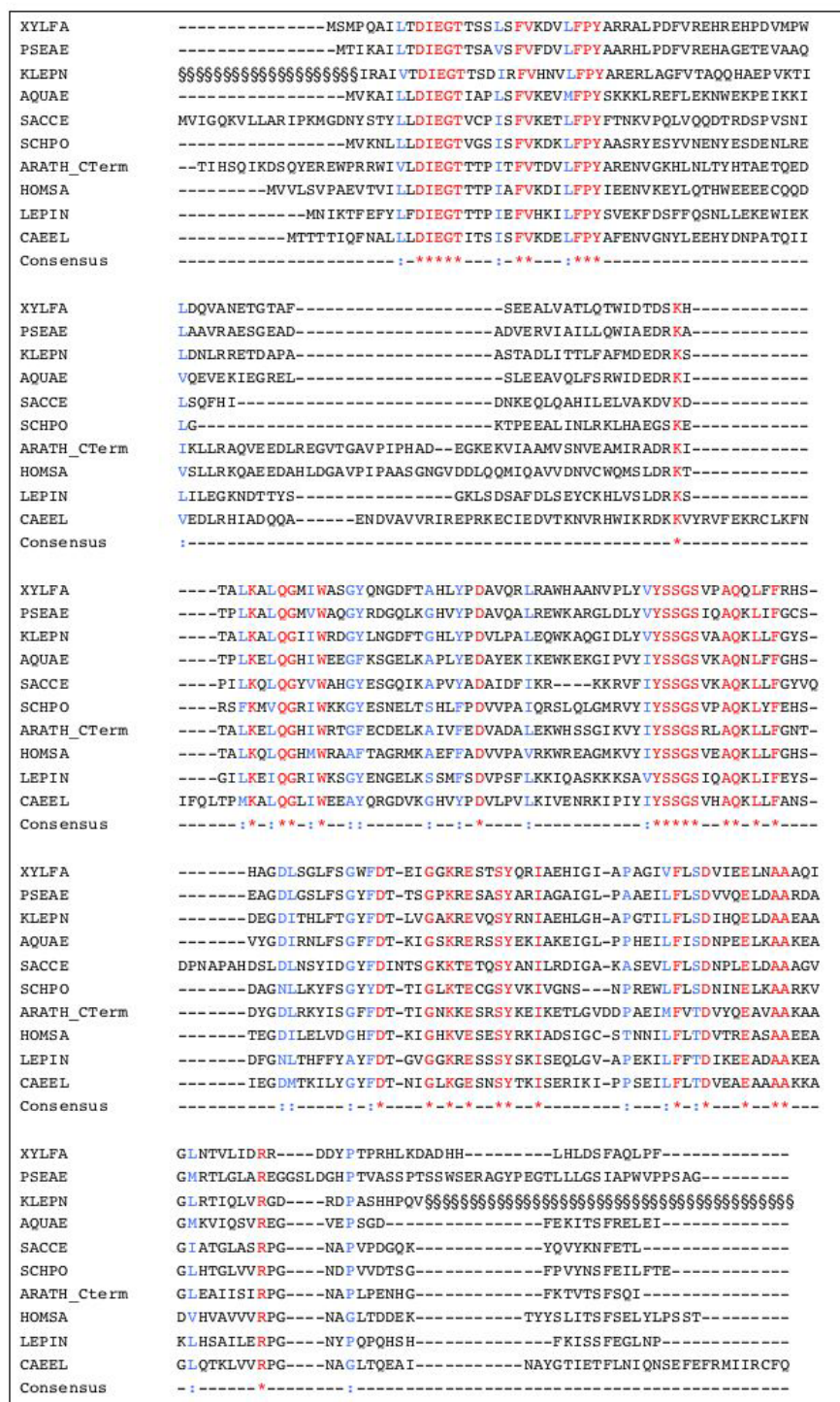


Figure 6
 Alignment of 2,3-diketo-5-methylthiopentyl-1-phosphate enolase-phosphatases. Important residues are indicated as in Figure 2. For *A. thaliana* (sequence from At5g53850) the C-terminal end of the protein is aligned. The § symbol at the beginning and the end of the *K. aeruginosa* sequence corresponds to regions that have not been identified from the ongoing genome programme sequences.



Figure 7

Alignment of aci-reductone dioxygenases. Important residues are indicated as in Figure 2. The N-terminus of the protein is poorly conserved.

Table 2: CO production by *Bacillus subtilis* on plates using GASTEC dosimeters.

Strain	Sulfur source used	
	MgSO ₄	MTR
WT 168	50.0	66.7
BFS1853 <i>mtnD::lacZ</i>	48.3	51.7
BFS4605 <i>yfIM::lacZ</i>	43.3	56.7

CO production is expressed in ppm/hour accumulated during 20 hours of incubation. Background is not subtracted. A difference of 5 in the limit of the palladium metal precipitate is reproducibly visible. The values given correspond to three replicates of each experiment. Note that it is not possible to distinguish between purely black and reddish black precipitates (often due to thiol containing gasses). Background precipitation in the absence of bacteria was of the order of 12.5 ppm/hour.

Finally, *mtnE* codes for KMTB aminotransferase, when it exists as a specific enzyme. The pathway with this nomenclature is summarized in Figure 1.

Discussion

The beginning of the methionine salvage pathway is variable, even in phylogenetically related organisms. It can either correspond to the tandem reactions, MTA nucleosidase/MTR kinase (as in *B. subtilis* or *K. pneumoniae*) or start with MTA phosphorylase as in *P. aeruginosa*, many Eukarya or presumably in *T. tengcongensis*, a Firmicute living at high temperature in partially anaerobic conditions. The pathway can further vary at the level of enolase, with either an enolase (RuBisCO-like) and a phosphatase (in *B. subtilis*, *B. cereus* and *B. anthracis*, for example), or, more often, with an enolase-phosphatase as in a variety of organisms, including many Bacteria (e.g. *P. aeruginosa*), Plants and Animals (Figure 1). An explicit substantiation of this latter pathway can be seen in *A. thaliana* where gene At5g53850 codes for a protein made of two domains, similar to KOG2631 and KOG2630, with proposed activities of the Class II aldolases and enolase-phosphatases. We do not discuss here the case of Archaea because, although there seems to be a clear counterpart of the methionine salvage pathway, starting from MTA phosphorolysis [27], no clearcut biochemical data are yet available, while the last step (dioxygenase) is probably missing or altered to another type of reaction (many Archaea are strict anaerobes).

In order to further support our description of the pathway we extrapolated from the *B. subtilis* data to other genomes, based on chromosome neighborhood [35] (Figure 8). Here we discuss four cases: *K. pneumoniae*, *P. aeruginosa*, *Xylella fastidiosa*, *L. interrogans* and *T. tengcongensis*.

Most of the methionine salvage pathway has been uncovered in *K. pneumoniae* by the biochemical studies of Abeles and coworkers [25,28,29]. It was therefore interesting to identify the genes corresponding to the pathway. A complete genome sequence is known but not publicly available, while the *K. pneumoniae* genome programme at Washington University is still ongoing. We could however identify the genes for a complete methionine salvage pathway, which begins as in *B. subtilis* (MTA nucleosidase followed by MTR kinase), but differs at the level of the enolase-phosphatase step (Figures 3, 4, 5, 6).

In contrast with the behavior of *K. pneumoniae*, *P. aeruginosa* codes for an MTA phosphorylase. Gene PA-3004, located downstream from *nagZ* (coding for N-acetyl-D-glucosaminidase) and perhaps from transcriptional regulator gene *psrA*, corresponds to *mtnP*. Operon *aroC*(PA-1681)-PA-1682-PA-1683-PA-1684-*masA*(PA-1685) is likely to code for the core of the pathway in which PA-1682 has an unknown transport function, whereas PA-1683(*mtnB*), PA-1684(*mtnD*) and PA-1685(*mtnC*) correspond to the enzymatic steps described above. Finally, *mtnA* corresponds to gene PA-3169, possibly the first gene of a large mixed-function cluster *mtnA-gyrA-serC-pheA-tyrB-aroE-cmk-rpsA-himD*. This cluster is also involved in biosynthesis of aromatic molecules, and the gene *tyrB* coding for a wide spectrum aminotransferase is probably involved in KMTB transamination, as is its counterpart in *K. pneumoniae* [34]. As a support to this analysis, both *mtnP* and *mtnB* mutants of *P. aeruginosa* could not grow on MTA as a sulfur source.

In *X. fastidiosa* a cluster of genes, presumably constituting an operon, is consistent with the existence of a complete methionine salvage pathway. This operon "*serA-xf-2207-xf-2208-xf-2209-xf-2210-xf-2211-xf-2212*" would code for glyceraldehyde-3-phosphate dehydrogenase (serine and one-carbon metabolism), subunits of a transporter, *mtnB*, *mtnD*, *mtnC* and a short fragment (N-terminus) of *mtnK*, respectively (Figure 3). A second gene cluster "*xf-2555-xf-2554-mtnA-gyrA-xf-2551*" codes for a tRNA synthetase, a protein of unknown function, *mtnA*, DNA gyrase and a protein of unknown function found in plants. There exists also a clear counterpart of *mtnP* (*xf-2353*, see Figure 2). This gene belongs to an operon (*xf-2358->xf-2352*), coding for an RNA methylase, a putative membrane protein highly conserved in cyanobacteria or bacteria associated to plants, a very short protein with similar sequence found in plants, a cell wall hydrolase, purine phosphoribosyltransferase, *MtnP* and a cold-shock-like protein. This operon is, at least in part, consistent with MTA and/or S-adenosylhomocysteine production and phosphorolysis. In particular, the first gene product would produce the latter molecule, while the phosphoribosyltransferase could scavenge adenine or a derivative.

Thus, *X. fastidiosa* has all genes needed for a functional methionine salvage pathway. The truncated form of *mtnK* suggests that the core metabolic genes might have been obtained by horizontal gene transfer [36], and that MtnK was useless in the presence of a functional MtnP. Presence of a gene coding for spermidine synthase (*xf-0143*) as well as a counterpart of AdoMet decarboxylase (*xf-1539*) indicates that the substrate, MTA, is indeed synthesized in *X. fastidiosa*. The similarity between the steps of the methionine salvage pathway in *K. pneumoniae*, *P. aeruginosa*, and *X. fastidiosa* suggests that similar pathways will be found in many gamma-proteobacteria.

Leptospira interrogans genome annotation has been particularly difficult because the majority of the genes present in this organism are only poorly related to known genes in other organisms. Indeed, the annotated genome contains a large number of small putative genes that may not really be coding DNA sequences [37]. This prompted us to explore the presence of a methionine salvage pathway in this organism. In order to have better chances to find the genes of the pathway, we started by looking for counterparts of the first gene in the pathway, *mtnA*. There is a clear counterpart, LA2868, in *L. interrogans* (Figure 3), as an isolated gene in chromosome I. The gene for the phosphorylase with clear features (molecular footprint) of MTA phosphorylase (Figure 2), *mtnP* (LA4248), is also present as an isolated gene in the same chromosome. In chromosome II, operon LB294-LB293-LB292-*fabG* codes for translation factor EFP, MtnD, MtnB and a putative oxoacyl-ACP-reductase. The last enzyme required for the pathway is the enolase-phosphatase (MtnC): it is encoded in an operon present in chromosome I "LA0931-LA0930-LA0929-LA0928". The first gene codes for a peptide that could have a regulatory role, the following gene codes for a permease, then we find *mtnC* (Figure 5). The operon ends with a gene coding for a short peptide that could have features of transcriptional regulators. This indicates that *L. interrogans* most probably has the complete methionine salvage pathway. Interestingly, this organism has two counterparts of spermidine synthase (one on each chromosome), enzymes that should result in the production of MTA. Gene LB312, on the second chromosome could be the counterpart of *speD* in this genome.

Finally, we tried to explore the pathway in *T. tengcongensis*, because although similar to *B. subtilis*, this organism thrives mostly under anaerobic conditions. In *T. tengcongensis* the phosphorylase would be encoded in a large cluster (either continuous or split into two operons: *tte-1584*<*tte-1594*) "*ileS-ssnA-araD-tdh2-gcn3-cbiO2-cbiQ2-pnp2-tte-1586*" upstream of "*metC3-tte-1584*". Both parts of this cluster, typical of a "sulfur island" [38], are preceded by a S-box riboswitch [20,21]. Because of the consistency of the functions encoded in this cluster it is

possible that *ileS* has been mistakenly thought to code for isoleucine tRNA synthetase, but rather codes for methionine tRNA synthetase (both enzymes are usually extremely similar). Because annotations are often propagated by similarity only, it would be most interesting to check experimentally whether this enzyme is an authentic methionine tRNA synthetase. If this were the case, this should prompt reanalysis and annotation of the whole class of cysteine, isoleucine, leucine, methionine and valine tRNA synthetases. The second gene could code for a metal-dependent hydrolase, probably hydrolyzing C-N bonds. The third gene, in the present context, is *mtnB*, *tdh2* could code for a metal-dependent enolase (differing from its counterpart in other organisms, hence tentatively named *mtnY*, but this has to be substantiated experimentally), *gcn3* is *mtnA*, the next two genes are divalent metal transporters, followed by *mtnP*, while the last gene could code for a component of a transporter. Thus the operon would be "S-box-*metS-ssnA-mtnB-tte-1591(mtnY)-mtnA-cbiO2-cbiQ2-mtnP-tte-1586*". For this organism, it is unlikely that a dioxygenase belongs to the pathway, because it lives mostly in the absence of oxygen. Indeed, we do not find a clear counterpart of *mtnD*. Starting from the end of the pathway we find that a broad-spectrum aminotransferase (labelled *hisC*, *tte-0380*) belongs to a large cluster mostly devoted to divalent-metal transport and synthesis of coenzyme B12, with two unknown genes *tte-0378* and *tte-0379*. Coenzyme B12 is often involved in pathways operating under anaerobic conditions and this should prompt further studies of the methionine salvage pathway under these conditions (*T. tengcongensis*, that belongs to Bacteria, might provide a missing link to explore the pathway in Archaea).

Sulfur metabolism has probably to be compartmentalized, because of the extreme reactivity of this atom towards dioxygen and radicals. In this respect, it is interesting to observe that, despite their apparent variety of origins, genes of the methionine salvage pathway have a tendency to cluster into operons (translation products of neighboring cistrons are produced in close proximity in the cell), or even fuse and give multidomain proteins (this is frequent in eukaryotes [39]). Another case of gene fusion that might be related to compartmentalization of sulfur containing molecules can be seen in *Aquifex aeolicus* where the MtnA protein begins with a domain of unknown function:

```
MDIRTHLGINQELSGKPKVVAEGYAEVLLKTQESMKAD-
DKGLVHGGFIFSAADYA
SMLAVNHPNVVLAGANVKFLKPKVKVGDDEIICKARVSED-
KGGKKKVLVECFKGEDKVFEGEFFTVFLID
```

that represents a whole COG domain, COG2050.1, PaaI. This domain has a signature for sulfur metabolite binding.

This further substantiates the idea that sulfur metabolism is compartmentalized.

Finally it was interesting to note that *B. subtilis*, when in the presence of significant amounts of MTR, was likely to produce CO, that may act as a gaseous mediator. This is the more interesting because *B. cereus* and *B. anthracis* possess the same methionine salvage pathway as *B. subtilis*. Furthermore, because this pathway exists in humans, it may be relevant to the ongoing discussion about the role of CO as a neuronal or gastro-intestinal mediator [40,41]. Analysis of similar arrangements in other genomes will lead to recognition of new features of the genetics of microbial sulfur metabolism.

Conclusions

The MTA => methionine salvage pathway is present in many Bacteria. This pathway provides us with a remarkable example of enzyme recruitment in bacteria associated to their biotope. Indeed the enolase-phosphatase step in the pathway is performed by enzymes of entirely different descent, RuBisCO being the most puzzling example. In the same way it seems quite remarkable that a family of GTP binding proteins, previously shown to be involved in translation elongation, universally belong to this pathway (MtnA). It will be an interesting link to compare the reactions in anaerobic environments. This will teach us how we could modify enzyme activities in the future and reprogram biochemical processes.

Methods

Genetic and biochemical experiments

Growth conditions

The following *B. subtilis* strains were used in this work: the wild type strain 168 [42], BFS1853 (*mtnD(ykrZ)::lacZ*) and BFS4605 (*yflM::lacZ*). These strains have been constructed during the *B. subtilis* functional analysis program (<http://locus.jouy.inra.fr/cgi-bin/genmic/madbase/progs/madbase.operl> and <http://bacillus.genome.ad.jp> [26,43]). *Escherichia coli* strains were grown either on LB or on nutrient yeast broth (NYB) liquid or agar plates at 37°C. *B. subtilis* was grown 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 at 37°C. To assay for CO production, ED minimal medium plates were used with either a MgSO₄ containing medium or a sulfur-free basal medium (where MgSO₄ was replaced by MgCl₂ at 2 mM concentration). In the latter case, 10 µl of the sulfur source under investigation (MTR, 200 mM stock solution) was applied onto paper discs deposited at the center of the plate, after bacteria had been uniformly spread on the surface of the plate. 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). *P. aeruginosa* strains were routinely grown in NYB or on nutrient agar plates at 37°C. When required, antibiotics were added to media at the following concentrations: tetracycline (Tc), 25 µg ml⁻¹ (*E. coli*) or 125 µg ml⁻¹ (*P. aeruginosa*); kanamycin (Km), 50 µg ml⁻¹ (*E. coli*); ampicillin (Ap), 100 µg ml⁻¹ (*E. coli*); spectinomycin (Sp), 25 µg ml⁻¹ (*E. coli*) or 1,000 µg ml⁻¹ (*P. aeruginosa*); erythromycin plus lincomycin, 1 µg ml⁻¹ and 25 µg ml⁻¹ (*B. subtilis*). To counterselect *E. coli* S17-1 donor cells in matings with *P. aeruginosa* for gene replacement, chloramphenicol was used at a concentration of 10 µg ml⁻¹; mutant enrichment experiments were performed with tetracycline at a final concentration of 20 µg ml⁻¹ and carbenicillin at a final concentration of 2,000 µg ml⁻¹. Strain PAO503 and its derivatives were cultivated in NYB, washed and plated out on minimal medium E; 50 µl of methionine, homocysteine or MTA (50 mM each) were then spotted onto sterile filters placed on the bacterial lawn. Growth around the filters was scored after 18 h of incubation. The optical density (OD) of bacterial cultures was measured at 600 nm.

DNA manipulation and cloning procedures

Small- and large-scale preparations of plasmid DNA were carried out by the cetyltrimethylammonium bromide (CTAB) method [44] and with JetStar columns (Genomed, Basel, Switzerland), respectively. Chromosomal DNA was purified from *P. aeruginosa* with phenol:chloroform (1:1) by a method described previously [45]. Restriction enzyme digestions, PCR, ligation, agarose gel electrophoresis, and electroporation were performed using standard procedures [46,47]. For Southern blotting, chromosomal DNA was digested overnight with the appropriate enzymes, electrophoresed on a 0.8% agarose gel and transferred to a Hybond-N nylon membrane (Amersham Biosciences). Labeling of DNA probes, hybridization and detection were performed using the digoxigenin (DIG) DNA Labeling and Detection Kit (Roche) according to the manufacturer's procedures. Nucleotide sequences of PCR-derived constructs were determined on both strands with a dye terminator kit (Perkin-Elmer product 402080) and an ABI Prism 373 sequencer.

Construction of *P. aeruginosa* mutant strains

For the construction of strain PAO6412 (*metZ9011 mtnB::Ω-Sp/Sm*), the *mtnB* gene (= PA1683) was PCR-amplified using PAO1 genomic DNA as a template and primers PA1683-1 (5'-AAAAGAATTCTGCACCT-GGAATCGCTGGG-3') and PA1683-2 (5'-AAAAGGATC-CCGGTCTCGTCCGAGGAAGACTTG-3'), tagged with *Eco*RI and *Bam*HI artificial sites (italics), respectively. The *mtnB*

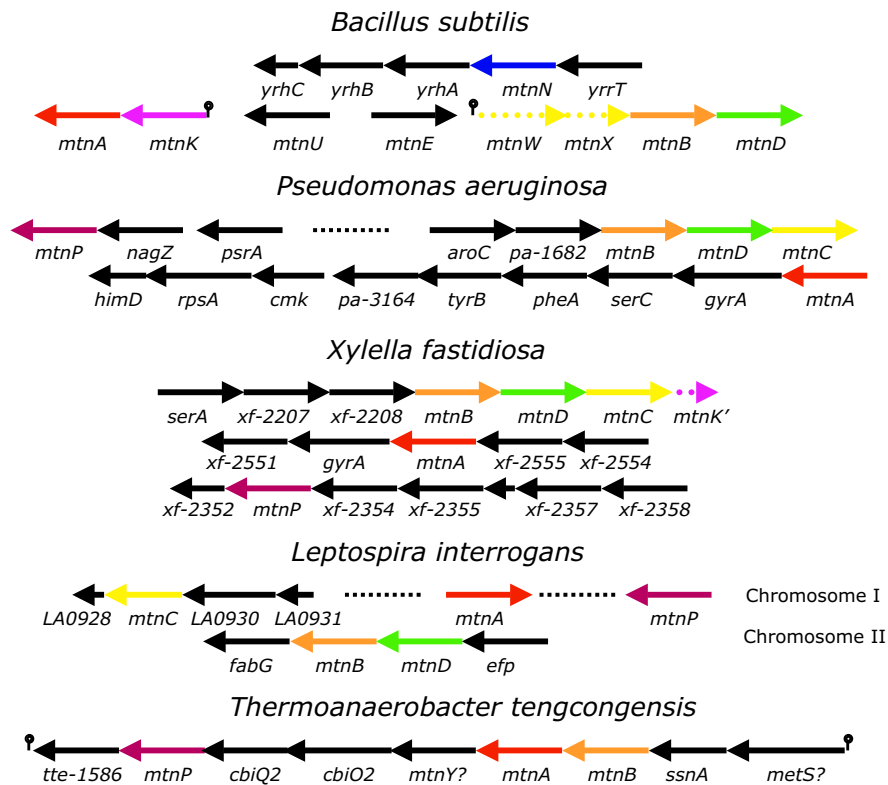


Figure 8

Variety of gene organisation for the methionine salvage pathway in different bacteria. The organisation of the genes coding for the methionine salvage pathway is shown in several bacteria: *B. subtilis*, *P. aeruginosa*, *X. fastidiosa*, *L. interrogans* and *T. tengcongensis*. The pathway is as described in Figure 1. Genes with the same function are indicated with the same color code. When a gene arrow is broken, this indicates that the function is the same, but that the gene product differs (enolase (MtnW) and phosphatase (MtnX) in *B. subtilis*, vs enolase-phosphatase (MtnC) in other organisms). The stem and loop symbol indicates the presence of a S-box riboswitch. In *B. subtilis* the enolase-phosphatase activity found in other organisms is replaced by two enzymes, a RuBisCO-like enolase and a phosphatase. In *X. fastidiosa*, downstream of the main operon one can see a truncated form of *mtnK* (broken arrow) requiring the presence of *mtnP* instead.

gene was interrupted by a spectinomycin-streptomycin resistance cassette (Ω -Sp/Sm) integrated as a 2-kb *Sma*I fragment into the *Sma*I site of *mtnB*; the resulting 3.3-kb *Eco*RI-*Xba*I fragment containing *mtnB*:: Ω -Sp/Sm was inserted into the suicide plasmid pME3087 [48] to generate pME3880. This plasmid was mobilized into PAO503 (*metZ9011*) by the donor *E. coli* S17-1 (*thi pro hsdR recA/RP4-2 Tc::Mu Km::Tn7*) and chromosomally integrated with selection for tetracycline resistance. Excision of the pME3087 carrying *mtnB* by a second crossover was obtained by enrichment for tetracycline-sensitive cells [49]. The tetracycline-sensitive, spectinomycin-resistant

clones (containing *mtnB*:: Ω -Sp/Sm) were checked by Southern blotting (data not shown).

For the construction of strain PAO6422 (*metZ9011 mtnP*:: Ω -Sp/Sm), the *mtnP* gene (= PA3004) was PCR-amplified with primers PA3004-1 (5'-AAAAGGATCCGTC-GAAGGCGGTTTCGAC-3') and PA3004-2 (5'-AAAAAAGCTTCATGTGGCGATTCCCGAAGAC-3') containing artificial sites for *Bam*HI and *Hind*III (italics). To generate pME3891, the 2-kb *Sma*I fragment containing the Ω -Sp/Sm cassette was integrated into the *Eco*RV site of *mtnP* and the resulting 3.3-kb *Kpn*I-*Xba*I fragment carrying

mtnP::Ω-Sp/Sm was inserted into the suicide plasmid pME3087. The resulting plasmid (pME3891) was delivered to the chromosome of PAO503 as described above, resulting in strain PAO6422, which was checked by Southern hybridization as above.

Preparation of MTR and CO assays

Methylthioribose (MTR) was prepared from MTA (Sigma, D5011) by acid hydrolysis as described by Schlenk *et al.* [50]. The CO production assay was carried using the GASTEC No.1DL dosimeters (Carbon Monoxide Low Range Passive Dosi-Tubes, Gastec Corporation, Japan). To detect CO production the ED minimal medium plates of 150 mm diameter were inoculated with bacteria and the dosimeter was activated by breaking the tube at its score. Plates were incubated at 37°C for 20 hrs and readings were recorded after the end of incubation period. Comparison of replicates of the same experiment (same volume of the growth medium, same temperature, same incubation time) indicated that the level of significance was better than 5 ppm. Control and MTR experiments were performed on the same day. It is not possible, however, to separate the nature of the different reducing gasses from the production of palladium metal precipitates. Replicates of the experiment on plates without bacteria gave an average level of reducing gasses of ca 12.5 ppm/h.

Computer-mediated analyses

Data were extracted from the microbial genome databases set up at <http://bioinfo.hku.hk/genolist.html>, using the following databases with their imbedded BLAST browsers and searching using appropriate patterns ("search patterns") when appropriate: AeuList, LeptoList, ThermaList and XyleList. For *B. subtilis*, the reference database was used: <http://genolist.pasteur.fr/SubtilList>. Analyses of domains were performed using "CD-search" at the NCBI [51]. Annotations were used with the help of SwissProt [52]. Alignments were performed using CLUSTALW and further refined manually [53].

Authors' contributions

All authors participated in the final form of the article. AS set up and performed the experiments meant to detect the presence of reducing gasses in *B. subtilis* and its mutants. VD performed the genetic experiments with *P. aeruginosa*. HA elaborated experiments of the methionine salvage pathway in *B. subtilis*. KM established the nomenclature and harmonized it with the Swissprot requirements. DH conceived the experiments with *P. aeruginosa*. AY established the biochemical protocol needed to identify the steps of the methionine salvage pathway in *B. subtilis*. AD performed the sequence comparisons and wrote the core of the article.

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