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Comparative genomics of the methionine metabolism in Gram-positive bacteria: a variety of regulatory systems

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ABSTRACT

Regulation of the methionine biosynthesis and transport genes in bacteria is rather diverse and involves two RNA-level regulatory systems and at least three DNA-level systems. In particular, the methionine metabolism in Gram-positive bacteria was known to be controlled by the S-box and T-box mechanisms, both acting on the level of premature termination of transcription. Using comparative analysis of genes, operons and regulatory elements, we described the methionine metabolic pathway and the methionine regulons in available genomes of Gram-positive bacteria. A large number of methionine-specific RNA elements were identified. S-boxes were shown to be widely distributed in Bacillales and Clostridia, whereas methionine-specific T-boxes occurred mostly in Lactobacillales. A candidate binding signal (MET-box) for a hypothetical methionine regulator, possibly MtaR, was identified in Streptococcaceae, the only family in the *Bacillus*/*Clostridium* group of Gram-positive bacteria having neither S-boxes, nor methionine-specific T-boxes. Positional analysis of methionine-specific regulatory sites complemented by genome context analysis lead to identification of new members of the methionine regulon, both enzymes and transporters, and reconstruction of the methionine metabolism in various bacterial genomes. In particular, we found candidate transporters for methionine (MetT) and methylthioribose (MtnABC), as well as new enzymes forming the S-adenosylmethionine recycling pathway. Methionine biosynthetic enzymes in various bacterial species are quite variable. In particular, *Oceanobacillus iheyensis* possibly uses a homolog of the betaine–homocysteine methyltransferase *bhmT* gene from vertebrates to substitute missing bacterial-type methionine synthases.

INTRODUCTION

Sulfur-containing amino acid methionine is synthesized *de novo* by most microorganisms and plants after the initial steps of inorganic sulfate assimilation and synthesis of cysteine or homocysteine (1). The fact that methionine is the universal N-terminal amino acid of proteins as well as the use of its derivative *S*-adenosylmethionine (SAM) in a variety of methyltransferase reactions argue for the importance of methionine in the cellular metabolism. There are two alternative pathways of methionine synthesis in microorganisms. The transsulfuration pathway of *Escherichia coli* involves cystathionine as an intermediate and utilizes cysteine as the sulfur source (2). In contrast, the direct sulfhydrylation pathway found in *Saccharomyces cerevisiae* (3), *Leptospira meyeri* (4) and *Corynebacterium glutamicum* (5) bypasses cystathionine and uses inorganic sulfur instead. While most microorganisms synthesize methionine via either one of these pathways, *C.glutamicum* utilizes both pathways (6).

Biosynthesis of methionine starts at homoserine, which is the common precursor for amino acids of the aspartate family, isoleucine, threonine and methionine (Figure 1). Homoserine is derived from aspartate semialdehyde by the *hom* gene product. Acylation of homoserine is catalyzed by homoserine acetyltransferase MetB in *Bacillus subtilis* (7) and by homoserine succinyltransferase MetA in *E.coli* (8), and these two enzymes are homologous. *L.meyeri* uses homoserine acetyltransferase MetX not related to the above enzymes (9). In the transsulfuration pathway, homocysteine is formed from *O*-acetylhomoserine or *O*-succinylhomoserine and cysteine in two steps, catalyzed by cystathionine γ-synthase MetI and cystathionine β-lyase MetC in *B.subtilis* (10). In the alternative pathway, *O*-acetylhomoserine is directly converted to homocysteine by *O*-acetylhomoserine sulfhydrylase MetY, utilizing sulfide as the sulfur donor (11).

Unlike the transsulfuration/sulfhydrylation enzymes that are present only in organisms with *de novo* methionine synthesis, methionine synthase is required by all organisms to ensure regeneration of the methyl group of SAM (1). Two types of methionine synthases can perform this function in *E.coli*. Reaction catalyzed by B_{12}-dependent protein MetH

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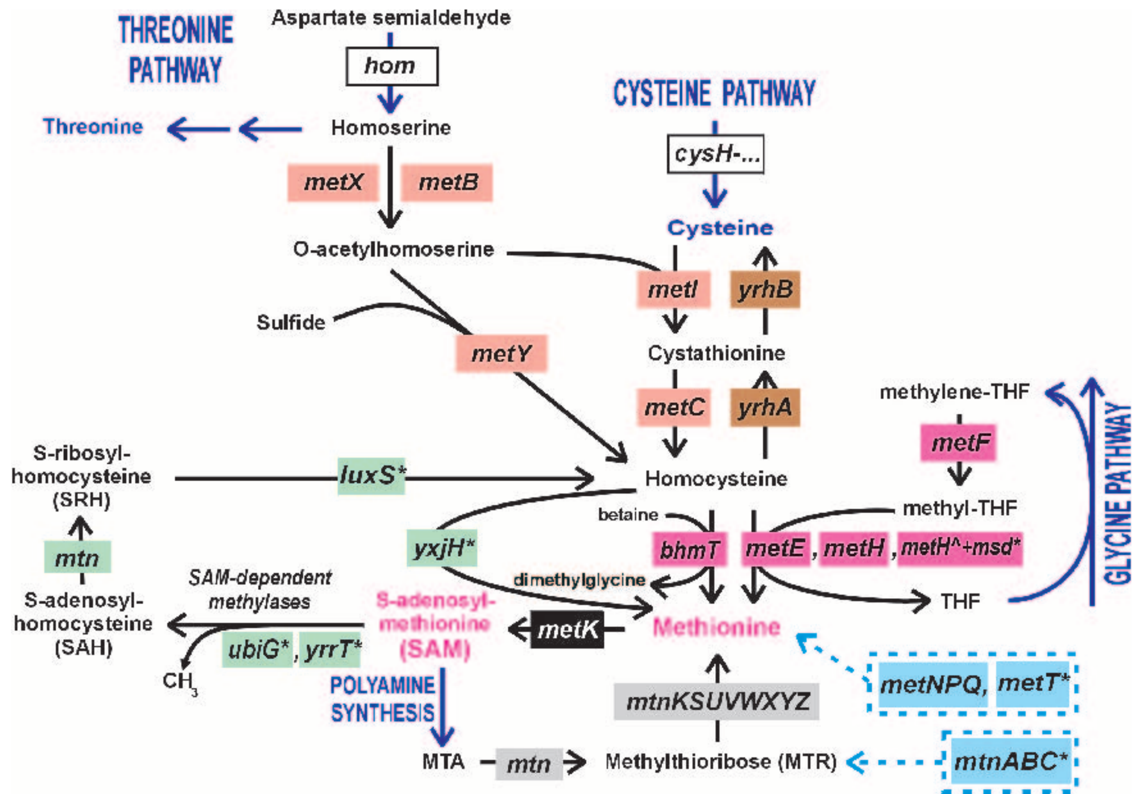

Figure 1. The methionine metabolic pathway in Gram-positive bacteria. The common gene names from *B.subtilis*, *E.coli* and *Leptospira interrogans* are used. Genes whose function was assigned in this study are indicated by asterisks. Different parts of the pathway are marked by colors: methionine biosynthesis, red and magenta; SAM synthesis, black; methionine salvage, gray; SAM recycling, green; reverse transsulfuration pathway for cysteine synthesis, brown; methionine and methylthioribose transport, blue.

with coenzyme B_{12} as a cofactor is more than 100-fold faster than the reaction catalyzed by B_{12}-independent isoenzyme, MetE (8). In enterobacteria, the methyl group of methionine is donated by methyl-tetrahydrofolate (methyl-THF). The latter is formed by reduction of methylene-THF in a reaction catalyzed by the *metF* gene product. *B.subtilis* has only the B_{12}-independent methionine synthase (formerly named MetC) and an ortholog of MetF, encoded by *yitJ*, that contains an extra N-terminal domain highly similar to the homocysteine-binding domain of MetH (12).

E.coli is able to uptake methionine by a high-affinity transport system encoded by the *metD* locus (8). Recently it was shown that this methionine transporter and its orthologs in various bacteria (*yusCBA* in *B.subtlis*, renamed *metNPQ*) constitute a new family of the ABC superfamily (13–16). No other methionine transporters have been identified in bacteria so far.

S-adenosylmethionine synthase, encoded by the *metK* gene, is responsible for formation of SAM from methionine and ATP. SAM is essential for a large number of methylation processes and is also used for modification of rRNA nucleotides and polyamine synthesis (17). The main product of the transmethylation reaction is *S*-adenosylhomocysteine (SAH). In *E.coli*, this molecule seems to be recycled to homocysteine in two steps: a nucleosidase encoded by the *pfs* gene (*mtn* in *B.subtilis*) hydrolyzes SAH into adenine and *S*-ribosylhomocysteine, which is then cleaved by a specific hydrolase to form homocysteine (18,19). Homocysteine can then be metabolized to synthesize cysteine and methionine. Notably, some organisms are able to synthesize cysteine from homocysteine via the reverse transsulfuration pathway (3,20). The cystathionine β-synthase and γ-lyase activities required for this pathway are possibly encoded by the *yrhA* and *yrhB* genes in *B.subtilis* (12,21). Methylthioadenosine is produced by the polyamine synthesis pathway and then hydrolyzed by a nucleosidase (encoded by *mtn* in *B.subtilis*), yielding methylthioribose (19). The latter is efficiently recycled in *B.subtilis* via the methionine salvage pathway encoded by the *mtnKSUVWXYZ* gene cluster (21–23).

Not methionine, but SAM, a major constituent of the intermediary metabolism, is the regulatory molecule for the methionine biosynthesis both in *E.coli* and *B.subtilis*. However, the mechanisms of SAM-dependent regulation differ in these bacteria. SAM-responsive repressor MetJ binds to tandemly repeated MET box sequences (5′-AGACGTCT-3′) and represses transcription of most *met* genes in *E.coli* (8). In *B.subtilis*, these genes are regulated by attenuation of transcription using a highly conserved regulatory leader sequence, the S-box (24,25). SAM directly and specifically binds to this RNA structural element, causing formation of the downstream terminator hairpin and subsequent premature termination of transcription (26–28). In the absence of effector molecules, formation of a more energetically favorable antiterminator, which is alternative both to the S-box domain and the terminator structure, leads to transcription read-through. The S-box domain and the terminator hairpin fold independently, while the

antiterminator structure consists of several conserved helices of the S-box and a new stem–loop which is alternative to the terminator and the base stem of the S-box. Moreover, Gram-positive bacteria have another control system involving premature transcription termination (T-box), which regulates expression of various aminoacyl-tRNA synthetases and genes involved in the amino acid biosynthesis (25,29,30). The T-box sequence is able to bind uncharged tRNA, promoting formation of the antiterminator. The major role in regulation is played by the T-box 'specifier codon', which interacts with the anticodon of an uncharged tRNA. As the position of this regulatory codon in the T-box structure is fixed, one can predict the amino acid specificity of the regulatory signal (31). For example, the presence of a T-box with an isoleucine specifier codon upstream of *ileS* in *Thermoanaerobacter tengcongensis* argues against a recently published theory that this gene is mis-annotated and encodes a methionyl-tRNA synthase (23).

Comparative genomics is a powerful technique for reconstruction of metabolic pathways and their DNA- or RNA-level regulation in bacteria (32–37). We analyzed the methionine pathway and its regulation in various Gram-positive bacteria. We extended the S-box and methionine-specific T-box regulons, that appear to be widely distributed in bacilli, clostridia and lactobacilli, identified a new regulatory signal for methionine biosynthetic and transport genes in streptococci, reconstructed the likely evolutionary scenario for methionine regulons of Gram-positive bacteria, and described the possible mechanism of dual regulation of the reverse transsulfuration pathway by cysteine and SAM in *Clostridium acetobutylicum* via RNA regulatory structures and antisense RNA. After reconstruction of the methionine metabolic pathways and regulatory interactions in Gram-positive bacteria, we identified several new enzymes and transporters involved in the methionine metabolism. In particular, we identified a set of candidate genes forming the complete SAM-recycling pathway.

MATERIALS AND METHODS

Complete and partial sequences of bacterial genomes were downloaded from GenBank (38). Preliminary sequence data were obtained also from the websites of The Institute for Genomic Research (http://www.tigr.org), University of Oklahoma's Advanced Center for Genome Technology (http://www.genome.ou.edu), the Sanger Centre (http:// www. sanger.ac.uk), the DOE Joint Genome Institute (http:// www. jgi.doe.gov) and the ERGO Database, Integrated Genomics, Inc. (39).

The conserved secondary structure of the S-box and T-box leaders was derived using the RNAMultAln program (A. A. Mironov, in preparation). This heuristic program simultaneously creates a multiple alignment and a conserved secondary structure for a set of RNA sequences using positional relationship of conserved sequence boxes and paired regions of candidate helices. This program is not based on the energy minimization, but rather uses simple heuristic rules of base pairing and analysis of complementary substitutions. The RNA-PATTERN program (40) was used to search for new S-boxes and methionine-specific T-boxes in bacterial genomes. The input RNA pattern described the RNA secondary structure and the sequence consensus motifs as a set of the following parameters: the number of helices, the length of each helix, the loop lengths and description of the topology of helix pairs. The latter is defined by coordinates of helices. For instance, two helices may be either independent or embedded helices, or else they could form a pseudoknot structure. This definition is similar to an approach implemented in the Palingol algorithm (41). Free energy of the S-box structures were calculated using Zuker's algorithm of free energy minimization (42,43) implemented in the Mfold program (http:// bioinfo.math.rpi.edu/~mfold/rna). RNA secondary structure of the antiterminator/antisequestor conformation, which includes parts of the S-box and overlaps with the terminator/ sequester hairpin (Figure 7A), was predicted using Mfold with the input including the S-box sequence extended downstream as far as the center of the terminator/sequestor hairpin. This structure is more energetically favorable than S-box in the absence of ligand. To model the effect of ligand binding that stabilizes the S-box, formation of the terminator/sequester hairpins was modeled with input sequence starting immediately downstream of S-box.

A simple iterative procedure implemented in the program *SignalX* was used for construction of the MET-box profile from a set of upstream gene fragments (44). Weak palindromes were selected in each region. Each palindrome was compared to all other palindromes, and the palindromes most similar to the initial one were used to make a profile. The positional nucleotide weights in this profile were defined as

$$W(b,k) = \log[N(b,k) + 0.5] - 0.25 \sum_{i=\mathrm{A,C,G,T}} \log[N(i,k) + 0.5],$$

where $N(b, k)$ was the count of nucleotide b in position k (45). The candidate site score was defined as the sum of the respective positional nucleotide weights:

$$Z(b_1 \ldots b_L) = \sum_{K=1 \ldots L} W(b_k, k)$$

where k was the length of the site. Z-score can be used to assess the significance of an individual site.

These profiles are used to scan the set of palindromes again, and the procedure was iterated until convergence. Thus a set of profiles is constructed. The quality of a profile was defined as its information content (46)

$$I = \sum_{K=1 \ldots L} \sum_{i=\mathrm{A,C,G,T}} f(i,k) \log[f(i,k)/0.25]$$

where $f(i, k)$ is the frequency of nucleotide i in position k of sites generating the profile. The best profile is used as the recognition rule.

Each genome was scanned with the profile, and genes with candidate regulatory sites in the upstream regions (in positions −325 to +25 relative to the translation start) were selected. The threshold for the site search was defined as the lowest score observed in the training set.

Protein similarity search was done using the Smith–Waterman algorithm implemented in the GenomeExplorer program (47). Orthologous proteins were initially defined by the best bidirectional hit criterion (48) and if necessary confirmed by construction of phylogenetic trees for the

corresponding protein families. The phylogenetic trees of the methionine biosynthesis and transport proteins were constructed by the maximum likelihood method implemented in PHYLIP (49). Multiple sequence alignments were done using CLUSTALX (50). Transmembrane segments were predicted using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). The COG (48), InterPro (51) and PFAM (52) databases were used to verify the protein functional and structural annotation.

Alignments of S-box and T-box sequences mentioned in this paper, as well as sequences of proteins whose functions were assigned here are available as supplementary Figures 1, 2 and 3, respectively.

RESULTS

Reconstruction of methionine regulons

Initially, orthologs of known methionine biosynthetic and transport genes (MET) were identified by similarity search in the genomes of all available Gram-positive bacteria (Table 1). For further analysis, positional clusters (including possible operons) of the MET genes were also described in this table. The *hom* gene for homoserine dehydrogenase shared by the methionine and threonine biosynthesis was considered only if it was co-localized or co-regulated with other MET genes.

S-box regulon. Then, we constructed the pattern of the S-box motif using the training set of 18 S-box leader regions (24), and scanned available genomic sequences using the RNA-PATTERN program. Multiple alignment of 100 S-box leaders from 23 bacterial genomes confirms high degree of conservation of the S-box primary and secondary structure (see supplementary Figure 1). Similar to other metabolite-binding RNA elements (32), the S-box motif has a set of helices (P2 to P5) closed by a single base stem (P1), and numerous highly conserved regions (Figure 2). In fact, the regions of sequence conservation cover most of the S-box sequence, and therefore, may be involved in SAM binding or tertiary interactions. Only two stem–loops at the ends of the fourth and fifth helices are not conserved on the sequence level and have variable length. The end loop of helix P3 (5′-CnGG-3′) possibly forms a pseudoknot structure with the interior loop between helices P4 and P5 (5′-CCnG-3′). This possible pseudoknot interaction is confirmed by several compensatory substitutions and could be required for the formation of stable S-box tertiary structure. For instance, the S-box structure on the *Bacillus cereus mtnW* gene contains a possible pseudoknot (5′-CGAG-3′; 5′-CTCG-3′) with complementary substitutions in the conserved positions of both arms.

Among Gram-positive bacteria, the S-box motif is widely distributed in the orders Bacillales and Clostridia, but it has not been found in Lactobacillales, including *Enterococcus*, *Streptococcus* and *Lactococcus* species (Table 1). Positional analysis of genes possessing this regulatory motif has showed that the S-box regulon in Gram-positive bacteria contains most genes of the methionine biosynthesis and transport, as well as the SAM synthase gene *metK*. The S-box regulon is most extensive in three bacilli, *B.subtilis*, *B.cereus* and *O.iheyensis*, where it contains 11, 16 and 13 regulatory elements, respectively, and includes additional genes for the cysteine biosynthesis and methionine salvage pathways, as well as hypothetical genes. The detailed phylogenetic and positional analysis of the S-box-regulated genes in Gram-positive bacteria is given in the next section.

The S-box motif is not restricted to the *Bacillus/Clostridium* group of bacteria. In two actinobacteria, *Streptomyces coelicolor* and *Thermobifida fusca*, S-box precedes the hypothetical gene *SCD95A.26*. In addition, single S-box motif was found upstream of the *metY-metX* operon in the genomes of *Chlorobium tepidum*, *Chloroflexus aurantiacus* and *Cytophaga hutchisonii*, as well as upstream of the *metNPQ* operon encoding methionine transport system in *Petrotoga miotherma*. A more relaxed search in (28) identified candidate S-boxes also in the genomes of *Fusobacterium nucleatum* (upstream of the *metK* and *metN* genes), *Deinococcus radiodurans* (*metH* and *metN*), *Xanthomonas campestris* (*metX*) and *Geobacter sulfurreducens* (*metB* and *metX*). All other bacterial taxonomic groups with available genomic sequences seem to lack the S-box regulatory system.

Recent experiments (26–28) demonstrated that SAM-dependent regulation of the methionine biosynthesis genes of *B.subtilis* involves attenuation of transcription using formation of alternative secondary structures in the S-box region. Here we tested whether the same regulatory mechanism could operate for all found S-boxes. Downstream of all S-boxes from the *Bacillus/Clostridium* group of bacteria we identified additional hairpins that are followed by runs of thymidines and therefore are candidate terminators of transcription. In addition, we observed complementary RNA regions that partially overlap both the proposed terminator and the base stem (helix P1) of the S-box. Thus, the same termination-antitermination S-box mechanism possibly operates in all Gram-positive bacteria.

Analysis of the upstream regions of the S-box-regulated genes from two actinobacteria reveals another possible mode of regulation. In this case the S-box motif directly overlaps the ribosome-binding site of the *SCD95A.26* gene, possibly acting as a sequestor. We predict that in these bacteria, SAM-stabilized S-box structure directly represses initiation of translation. Similar mode of regulation was previously suggested for other metabolite-responsive riboswitches in actinobacteria (32).

Methionine-specific T-box regulon. Using a training set of experimentally known T-box structures from *B.subtilis* and other Gram-positive bacteria (31), we constructed a pattern for the T-box motif and scanned bacterial genomes using the RNA-PATTERN program. The pattern was absolutely specific since candidate T-boxes were mostly found upstream of amino acid metabolism-related genes (data not shown). Then we selected 38 T-box sequences with AUG (methionine) specifier codons (see supplementary Figure 2 for multiple alignment). Although the T-box system is widely used in bacteria from the *Bacillus/Clostridium* group (where more than 200T-boxes were identified; A. G. Vitreschak, unpublished results), methionine-specific T-boxes occur only in a limited number of species.

In the genomes of *Bacillus halodurans* and six clostridia, there is only one methionine-specific T-box that precedes the *metS* gene encoding methionyl-tRNA synthetase (not included in Table 1). The methionine T-box regulation seems to be extensively used only in the Lactobacillales group (30 Met-T-boxes), where it exclusively controls methionine biosynthesis and transport genes (Table 1). This phylogenetic

Table 1. Methionine biosynthesis and transport genes and methionine-specific regulatory elements in Gram-positive bacteria

Genome	AB	Methionine biosynthetic genes	MetK	Transporters	Other genes
Bacillales:					
Bacillus subtilis	BS	*metB*; *S-metI-metC*; *S-metF^*; *S-metE*; *S-cysH-ylnABCDEF*	*S-metK*	*S-metNPQ*; *metQ2*	*mtnZYXW S-mtnV<>mtnU S-mtnKS;S-yoaD*; *yrrT-mtn-yrhAB*; *luxS*; *S-yxjH1-S-yxjH2*
Bacillus cereus	BC	*S-metY-metB-hom*; *metC-metI-S<>S-metF^-metH*; *S-cysH-ylnBCADEF*; *metX*; *B-metE*	*S-metK*	*S-metNPQ1-metQ2*; *S- metNPQ3*; *S-metQNP4*; *S-metT*; *S-mtnABC*; *S-tomBCDFA*; *S-mtsABC*	*mtnZYXW S-mtnV<>mtnU S-mtnKS*; *yrrT-mtn-yrhAB*; *luxS*; *S-mdh*; *S-hmrA*
Bacillus halodurans	BH	*metY*; *metB*; *S-hom*; *S-metI-metC-metF^-metH*;*B-metE*	*S-metK*	*S-metNPQ*	*S-BH0835*; *mtn*; *luxS*
Bacillus stearothermophilus #	BE	[*metY*; [*metB*; [*S-metI-metC*; [*S-metF^-metH*	*S-metK*	*S-metNPQ*; *mtnABC*	*mtnZYXW S-mtnV<>mtnU S-mtnKS*; *yrrT-mtn-yrhAB*; *luxS*
Oceanobacillus iheyensis	OB	*S-metY1*; *metB*; *S-bhmT*; *S-X-metY2*	*S-metK*	*S-metNPQ1*; *S-X-metQNP2*; *S-metNPQ3*; *S-metNPQ4-hmrA*	*yrrT-luxS–yrhAB*; *mtn*; *S-yxjH*; *S-OB1276*; *OB3079-S<>S-OB3078*; *S-OB2779-78*
Staphylococcus aureus	SA	*S-metX*; *T-metI-metC-metF^-metE-mdh*	*S-metK*	*S-metT*; *S-metNPQ1*; *hcp-mtsABC*	*yrhAB-metNPQ2*; *luxS <>hmrA*; *mtn*
Listeria monocytogenes	LMO	*S-metY-metX*; *S-metE-metI-metC-metF^*	*S-metK*	*S-metNPQ1*; *S-metQNP2*; *S-tomABCDF*	*S-yxjH*; *mtn*; *luxS*
Lactobacillales:					
Enterococcus faecalis	EF	no	*metK*	*T-metNPQ1*;*T-T-metNPQ2*;*metNPQ3*;*T-tomA*; *mtsABC*	*T-yxjH*; *luxS*; *mtn*
Lactobacillus plantarum	LP	*T-metB-metY-hom*; *T-metI*; *T-metE- metF^*	*metK*	*T-metNPQ1*; *T-metNPQ2*	*T-yxjH1*; *yxjH2*; *luxS*; *mtn*
Lactobacillus gasseri #	LGA	No	?	*T-metQNP*; *hcp-mtsABC*	*T-yxjH-luxS*; *mtn*
Lactobacillus casei #	LCA	*T-metE-metF*	*metK*	*T-metQ1*; *T-metQ2-metNP*	*T-yxjH-luxS*; *mtn*
Lactobacillus delbrueckii #	LDB	*metY*; *metB-luxS1-metQ2*; *T-luxS2-metE-metF*	*metK*	*T-metNPQ*; *hcp-mtsABC*	*mtn*
Lactobacillus brevis #	LB	no	?	*T-metNPQ*	*T-yxjH*; *luxS*; *mtn*
Oenococcus oeni #	OOE	*metY*; *yxjH2-metI-metC-metB*	*metK*	*T-metNPQ1*;*T-metQ2-yxjH1-hmrB-metNP*; *mtsABC*	*yxjH3*; *luxS*; *mtn*
Leuconostoc mesenteroides #	LME	*T-metB-metI-metC-yxjH1-luxS -T-yxjH2-metY*; *T-metF-E*	*metK*	[*metQ1-2-hmrB-metNP*; *metQ3*; *T-hcp-mtsABC*	*mdh1*; *T-mdh2* *mtn*
Pediococcus pentosaceus #	PPE	no	*metK*	*T-metNPQ*	*T-yxjH*; *luxS*; *mtn*
Streptococcaceae:					
Lactococcus lactis	LL	*metY*; *metB-metI*; *M-metE-metF*	*metK*	*metQ1-metQ2-metQ3-metQ4-metNP-mtsABC*	*yxjH*; *luxS*; *mtn*
Streptococcus agalactiae	SAG	*M-metE-metF^*	*metK*	*M-metQ-hmrB-metNP-/-mtsABC*; *M-metQ2*; *metNPQ3*	*luxS*; *mtn*
Streptococcus mutans	MN	*M-metY-mdh*; *M-M-metB*; *metI*; *M-M-metE-metF^*	*metK*	*M-metQ-hmrB-metNP-X-M-hcp-mtsABC*	*M-yxjH*; *luxS*; *mtn*
Streptococcus pneumoniae	PN	*M-metY*; *M-metB*; *M-metI*; *M-metE-metF*	*metK*	*M-metQ-hmrB-metNP*; *M-hcp-mtsABC*	*M-fhs*; *M-folD*; *luxS*; *mtn*
Streptococcus pyogenes	ST	*metC*	*metK*	*M-metNPQ*	*luxS*; *mtn*
Streptococcus suis #	SSU	*M-metY*; *metB*; [*metI*; *M-metE-metF*	*metK*	[*metQ-hmrB-metNP*]; [*hcp-mtsABC*	*M-yxjH*; *M-mdh*; *luxS*
Streptococcus thermophilus #	STH	*metY*; *M-M-metB*; *M-metI*; *M-M-metE-metF*	*metK*	*M-metQ-hmrB-metNP-X-hcp-mtsABC*; *metQ2*	*M-yxjH*; *M-mdh*; *luxS*; *mtn*
Streptococcus uberis #	SUB	no	*metK*	*M-metQ-hmrB-metNP*; *M-hcp-mtsABC*	*luxS*
Clostridia:					
Clostridium acetobutylicum	CAC	*S-metY*; *S-metB*; *S-metI-metC*; *metF^*; *S-metH*	*S-metK*	*S-metNPQ*	*Tc-ubiG-yrhBA><-S*; *luxS*; *mtn*
Clostridium perfringens	CPE	no	*S-metK*	*S-metT*; *mtsABC*	*ubiG-yrhBA-luxS*; *mtn*
Clostridium botulinum	CB	*S-metY-hom-metB*; *S-metF-msd-metH^*	*S-metK*	*S-metT*; *S-metNPQ1-metQ2*; *metNP2*	*ubiG-yrhB-luxS-yrhA*; *mtn*
Clostridium tetani	CTC	*S-metY-metB*; *-msd-metH^*	*S-metK*	*S-metT*; *S-metNPQ*	
Clostridium difficile	DF	*S-metY-metB*; *S-hom*; *folD-X-metF*; *luxS-msd-metH^*	*S-metK*	*S-metNPQ1*; *S-metQ2*	*mtn*
Thermoanaerobacter tengcong.	TTE	*S-hom-metY-metX*; *S-metF-metH^*	*S-metK*		
Streptomyces coelicolor	SX	*metF*; *B-metE*; *metH*	*metK*	*metNPQ*	*S-SCD95A.26*
Thermobifida fusca #	TFU	*metY-metX*; *metF*; *metH*	*metK*	*metNPQ*	*S-SCD95A.26*
Chlorobium tepidum	CL	*S-metY-metX*; *metF*; *metH*	*metK*		
Chloroflexus aurantiacus	CAU	*S-metY-metX*; *metF*; *metH*	*metK*		
Cytophaga hutchinsonii	CHU	*S-metY*; *metX*; *metE*; *metH--metF*	*metK*		
Thermotogales (*TM, PMI*)		*metY-metB*; *metF-msd-metH^*	*metK*	*S-metNPQ*	
Fusobacterium nucleatum	FN	no	*S-metK*	*S-metNPQ*	
Deinococcus radiodurans	DR	*metY-metX*; *S-metH-X-metF*	*metK*	*S-metNP-metQ1-metQ2*	

Genes of the upper part of the methionine pathway involved in the homocysteine synthesis are shown in red, methionine synthases and methylene-THF reductases, in magenta. Genes encoding transport proteins are shown in blue. Genes from the SAM recycling and reverse transsulfuration pathways are in green and brown, respectively. Genes forming one candidate operon (with spacer less than <100 bp) are separated by dashes ('–'). Direction of transcription in divergons is shown by angle brackets. Predicted S-boxes ('S'), methionine-specific T-boxes ('T'), MET-boxes ('M') and B12-elements ('B') are shown in yellow, green, red, and blue, respectively. Cysteine-specific T-box ('Tc') is also shown in green. Contig ends are marked by square brackets. Genome abbreviations are given in column 'AB' with unfinished genomes marked by a hash.

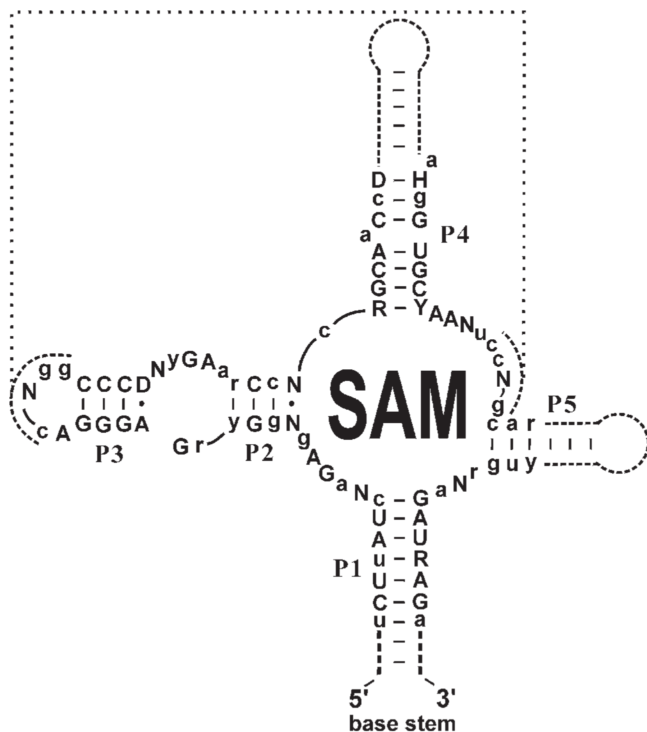

Figure 2. Conserved S-box structure. Capitals indicate invariant positions. Lower case letters indicate strongly conserved positions. Degenerate positions: R = A or G; Y = C or U; D = A, G or U; H = A, C or U; N = any nucleotide. Conserved helices are numbered P1 to P5. Dashes and dots indicate obligatory and facultative base pairs, respectively. Stem–loops of variable lengths are shown by broken lines. Possible tertiary interaction between the end loop of helix P3 and the interior loop is shown.

distribution is consistent with the absence of the S-box regulatory system in all available genomes of Lactobacillales (see Discussion). Among all other available bacterial genomes, we have found only one additional methionine T-box. It precedes the *metICFE-mdh* operon in *Staphylococcus aureus*, a bacterium that mainly uses the S-box system.

Methionine regulation in streptococci. Then we attempted to analyze potential methionine regulons in Streptococcaceae, a large family of Gram-positive bacteria, which have neither S-boxes nor methionine-specific T-boxes. For this aim, we collected upstream regions of all MET genes from *Lactococcus lactis* and *Streptococcus* species and applied the signal detection procedure. A highly conserved 17 bp palindromic sequence (named MET-box) with consensus 5′-TATAGTTtnaAACTATA-3′ was identified in all streptococci (Table 2). To find new members of the candidate regulon, the derived profile for the MET-box signal was used to scan the genomes. In various *Streptococcus* species, the MET regulon appears to include most methionine biosynthesis and transport genes, as well as several hypothetical genes including *yxjH*, *mdh*, *fhs* and *folD* (Table 1). The predicted MET regulon in *L.lactis* contains only one transcriptional unit, the *metEF* operon. Given the palindromic structure of the derived signal, typical to binding signals of transcriptional regulators, we propose that the MET-box signal plays a role in transcriptional regulation of the methionine metabolism in *Streptococcus* species and *L.lactis*, although the responsible regulatory protein

Table 2. Candidate methionine-specific DNA signals (MET-boxes) in Streptococcaceae

Gene	Pos	Score	Sites
consensus:			TATAGTTtnaAACTATA
Lactococcus lactis			
metE	−90	6.51	TATAGTTTAAAACTATA
Streptococcus agalactiae			
metQ1	−98	6.51	TATAGTTTAAAACTATA
metE	−131	6.51	TATAGTTTAAAACTATA
metQ2	−116	5.87	TATAGTTaAAAgCTATA
Streptococcus mutans			
yxjH	−95	6.51	TATAGTTTAAAACTATA
metY	−65	6.27	TATAGTTTAtAACTATA
metE	−156	6.11	TATAGcTTAAAACTATA
	−187	5.73	TATAGaTgAAAACTATA
metQ	−99	5.87	TATAGTTTTtAgCTATA
metB	−4	5.81	TATAtTTgAtAACTATA
	−38	5.47	TATAGTTggAAcCTATA
hcp	−109	5.43	TATAGgTaAtAACTATA
Streptococcus pneumoniae			
metQ	−100	6.51	TATAGTTTTAAACTATA
metE	−100	6.25	TATAGTTTcAAACTATA
fhs	−155	6.25	TATAGTTTcAAACTATA
metI	−92	6.25	TATAGTTTgAAACTATA
folD	−99	6.11	TATAGTTTAAAgCTATA
metB	−108	5.95	TATAaTTgAAAACTATA
metY	−208	5.65	gATAtTTgTAAACTATA
hcp	−65	4.92	TATAaggTAAAACTATA
Streptococcus pyogenes			
metQ	−104	5.91	TATAGTTTAAAACTATg
Streptococcus suis			
metY	−93	6.09	TATAGTTgTtAACTATA
yxjH	−99	5.67	TATAGTTTTtAACTATg
mdh	−80	5.26	TATAGTTaAAAgCaATA
metE	−100	4.88	TATAGTcaAAAgtTATA
Streptococcus thermophilus			
metE	−98	6.51	TATAGTTTAAAACTATA
	−170	5.63	TATAGTTaTtAgCTATA
metQ	−104	6.27	TATAGTTaAAAACTATA
yxjH	−318	5.90	TATAGTcTAAAACTATA
metB	−101	5.73	TATAGTTTAAAgtTATA
	−67	5.20	TATAtTTgAtAACaATA
mdh	−141	5.71	TATAGcTTAAAgCTATA
metI	−128	5.66	TATAGTaaTAAACTATA
Streptococcus uberis			
metQ	−102	6.11	TATAGTTTTAAgCTATA
hcp	−86	5.90	TATAGTTTTAgACTATA

Site positions in ‘Pos’ column are given relative to the translation start site.

remains to be identified. Interestingly, in a recent study it was shown that the LysR-type transcriptional regulator MtaR is necessary for the efficient methionine uptake in *Streptococcus agalactiae* (53). MtaR has orthologs in other studied *Streptococcus* species as well as in *L.lactis*. Since we have found MET-boxes upstream of the predicted methionine transport operons in *S.agalactiae*, we suppose that this MET-box is the DNA binding signal of MtaR. Moreover, the MET-box signal is similar to signals recognized by LysR-family regulators in length and palindromic symmetry.

Reconstruction of the methionine pathway

Positional analysis of a large number of regulatory elements (S-, T- and MET-boxes) in Gram-positive bacteria allowed us to identify new genes possibly involved in the methionine

metabolism. In addition, we analyzed the candidate MetJ binding sites upstream of some methionine synthesis and transport genes in gamma proteobacteria (data not shown). The detailed analysis of new members of the methionine regulons and reconstruction of the metabolic pathways in various organisms is presented below.

Methionine biosynthesis. The pathway of methionine biosynthesis via the transsulfuration or direct sulfhydrylation route is conserved in most Gram-positive bacteria, but some steps vary. Only complete genomes of *F.nucleatum*, *Clostridium perfringens*, *Enterococccus faecalis*, *S.agalactiae* and *Streptococcus pyogenes*, as well as unfinished genomes of several lactobacilli and *Streptococcus uberis*, lack the *de novo* synthesis pathway but possess the SAM synthase gene *metK* (Table 1). We suggest that this metabolic gap could be filled by methionine-specific transport systems detected in these genomes (*metNPQ* or *metT*, see below).

We observed several cases of expansion of the methionine regulon, when the upstream reactions preceding the methionine biosynthetic pathway become methionine regulated. One example is homoserine dehydrogenase (the *hom* gene product in *B.subtilis*), which is shared by the threonine and methionine pathways (Figure 1). In most Gram-positive bacteria the *hom* gene is co-localized with the threonine biosynthesis genes *thrBC* (data not shown). However, in the genomes of two bacilli, three clostridia and *Lactobacillus plantarum*, we have found a second *hom* paralog belonging to the methionine regulon, either S-box or T-box (Table 1). Another example is the cysteine biosynthesis gene cluster (*cysH-ylnABCDEF*), which is a member of the methionine S-box regulon in *B.subtilis* and *B.cereus*, but not in other Bacillales. Interestingly, even in these two related bacteria, the *in vitro* affinities of candidate *cysH* S-box motifs to the effector molecule (SAM) vary by two orders of magnitude (28). Furthermore, the expression of the *B.subtilis cysH* operon is not repressed by methionine *in vivo*, indicating disfunction of the *cysH* S-box element (54). Indeed, this S-box contains a unique C→A substitution destabilizing helix 3. Moreover, upstream regions of *cysH* genes of other closely related genomes (*B.halodurans*, *Bacillus stearothermophilus*, *O.ihyensis*) demonstrate residual similarity to upstream regions of *B.subtilis* and *B.cereus cysH*, but cannot fold into the S-box structure (data not shown). In any case, in *B.cereus*, this functional regulatory interaction seems to be rational since the bacillary transsulfuration pathway of the methionine biosynthesis uses cysteine as a sulfur donor.

The first step of the methionine biosynthesis is catalyzed by one of two non-homologous homoserine *O*-acetyltransferases, MetB or MetX. The main difference of these two isoenzymes is that, in contrast to MetX from *L. meyeri*, the MetB enzyme from *B.subtilis* is feedback inhibited by SAM (7,9). The MetB isoenzyme detected in most Gram-positive bacteria possessing the methionine pathway is replaced by MetX in *Staphylococcus*, *Listeria* and *T.tengcongensis*, whereas the *B.cereus* genome encodes both proteins (Table 1). Phylogenetic distribution of the MetB and MetX isoenzymes differs significantly: the former prevails in enterobacteria, firmicutes and cyanobacteria whereas the latter is common in other proteobacteria, actinobacteria, various early branching bacteria and in fungi. Notably, the SAM-inhibited isoenzyme MetB is not methionine regulated in many cases, at least not using S-, T-, or MET-boxes (e.g. in *B.subtilis*). In contrast, the MetX synthesis is S-box controlled with the exception of *B.cereus* where both enzymes are present.

Then we have analyzed distribution of the direct sulfhydrylation and transsulfuration pathways of the methionine biosynthesis, which are catalyzed by the MetY and MetI-MetC, respectively. Among Gram-positive bacteria, the former prevails over the latter: twelve species have only MetY, two species have MetI-MetC, whereas seven remaining methionine-producing bacteria possess both pathways (Table 1). The role of single *metI* genes (not accompanied by *metC*) in several genomes containing the *metY* gene is still not clear. Thus, in contrast to *B.subtilis* and *S.aureus*, other methionine-producing firmicutes potentially use the direct sulfhydrylation pathway and, therefore, they do not require cysteine for the methionine synthesis.

Two non-homologous methionine synthases, coenzyme B_{12}-dependent MetH and B_{12}-independent MetE, are known in bacteria. The *metE* gene was detected in all methionine-producing firmicutes, except clostridia and two bacilli that have the *metH* gene. *B.cereus* and *B.halodurans* have both the B_{12}-dependent methionine synthase MetH, which belongs to the S-box regulon, and the B_{12}-independent isoenzyme MetE, that is likely regulated by coezyme-B_{12} via the *B12*-element riboswitch (34). The only exception is *O.iheyensis*, which has neither *metE*, nor *metH*. Analysis of candidate S-box signals allowed us to identify a new member of the methionine regulon in this bacterium, OB0691, which is similar to the betaine–homocysteine methyltransferase BhmT from mammals (55). BhmT catalyzes conversion of homocysteine to methionine, like the bacterial MetE and MetH isoenzymes, but it uses another methyl donor, betaine, instead of methyl-THF. Thus, we predict that *O.iheyensis* uses a eukaryotic-type methionine synthase and does not require the methylene-THF reductase MetF, which is absent in this bacterium.

B_{12}-dependent methionine synthases from clostridia and Thermotogales lack the C-terminal domain which is involved in reactivation of spontaneously oxidized coenzyme B_{12}, and therefore is required for the catalysis (denoted *metH^* in Table 1). In all these bacteria, except *T.tengcongensis*, we found a hypothetical gene located immediately upstream of the *metH^* gene (see *Thermotoga maritima TM0269* as a representative of this gene family). This gene, named *msd*, has no homologs in other genomes. However, we identified a conserved sequence motif (hhhThG-28-hEhhh[DE]-//-RxxxGY-32-Pxx[SA][TV]x[GA]hh, where 'h' denotes any hydrophobic amino acid), which is common to all full-length MetH proteins and to the Msd proteins. As shown by (56), the RxxxGY motif is critical for binding of SAM to the C-terminal reactivation domain of methionine synthase MetH. Thus, we tentatively assign the missing function of reactivation of the B_{12}-dependent methionine synthase to the product of the *msd* gene. The three-dimensional structure of the TM0269 protein was recently resolved in Joint Center for Structural Genomics (http://www.jcsg.org/), but this did not lead to assignment of a cellular role for this protein.

The *metF* gene encoding methylene-THF reductase has been identified in most Gram-positive bacteria that have methionine synthases (MetH or MetE), an exclusion being *Clostridium tetani*. The MetF proteins from Bacillales,

C.acetobutylicum, *L.plantarum* and two streptococci have an additional N-terminal domain highly similar to the homocysteine-binding domain of MetH (denoted *metF^* in Table 1). Previously, it was proposed that this domain could be involved in positive allosteric regulation of MetF by homocysteine (12).

Recycling of methylene-THF from THF is connected with interconversion of serine and glycine mediated by GlyA, belonging to the methionine regulon in *E.coli* (57). The *glyA* gene is present in all Gram-positive bacteria, but never in the methionine regulon. The alternative pathway of methylene-THF recycling, which proceeds in two steps and requires ATP and NADP, is mediated by FolD and Fhs. The corresponding genes are candidate members of the MET-box regulon in *Streptococcus pneumoniae*, whereas the *folD* and *metF* genes form one possible operon in *Clostridium difficile* (Table 1). On the other hand, the *folD* gene is a candidate member of the purine regulon in some γ-proteobacteria (58), that is also rational since methylene-THF is required for the purine biosynthesis. These facts once again demonstrate genome-specific regulon expansions, and, in particular, indicate considerable variability in regulation of the methylene-THF synthesis in bacteria.

Methionine transport. The only known transport system for methionine is the ABC transporter MetNIQ of enteric bacteria, which belongs to the methionine uptake transporter (MUT) family (13–15). An ortholog of this system in *B.subtilis*, encoded by the *metNPQ* operon, is regulated by the S-box system (12). Recently it was shown that *metNPQ* encodes an ABC permease transporting methionine sulfoxide, D-, and L-methionine (16). To describe candidate methionine transporters in Gram-positive bacteria, we combined similarity search for the *metNPQ* orthologs with identification of methionine-specific regulatory sites and with positional analysis of genes.

Candidate methionine transporters *metNPQ* are widely distributed in Gram-positive bacteria: they are absent only in two clostridia species (Table 1). In most cases, components of methionine transporters are encoded by clusters of co-localized genes which are preceded by S-boxes (in Bacillales and clostridia), methionine-specific T-boxes (in lactobacilli) or MET-boxes (in streptococci). To analyze the possible origin of a large number of *metNPQ* paralogs in Gram-positive bacteria, we constructed the phylogenetic tree for the substrate-binding components of MUT-family transporters (Figure 3). Though in some cases, e.g. in *L.lactis*, *Campylobacter jejuni*, *metQ* paralogs possibly result from recent genome-specific duplications, in most other cases they have diverged early. Since almost all large branches of the tree contain members of various methionine regulons, including MetJ-regulated orthologs from enterobacteria (15), we believe that the methionine specificity has been retained by all members of the MUT family. However, we cannot exclude the possibility that some members of the MUT family have a more broad specificity.

A new type of candidate methionine transporters, named MetT, was identified in some bacteria from the *Bacillus*/*Clostridium* group and γ-proteobacteria. Among Gram-positive bacteria, only *B.cereus*, *S.aureus* and three clostridia species have the *metT* genes, which are regulated by upstream S-boxes in all cases (see Table 1 and Figure 4 for genomic identifiers). Among γ-proteobacteria, only *Vibrio* and *Shewanella* species have *metT* genes, and they are preceded by candidate MetJ sites (data not shown). MetT proteins contain eleven predicted transmembrane segments and are similar to proteins from the NhaC Na+:H+ antiporter superfamily. Existence of likely *S*-adenosylmethionine-regulated *metT* in the complete genome of *C.perfringens* that has no methionine biosynthesis genes suggests that MetT is a methionine transporter.

The phylogenetic tree of transporters from the NhaC superfamily consists of four deeply diverged branches (Figure 4). The first branch comprises predicted lysine transporters LysW, most of which are preceded by regulatory *LYS*-elements (35). The second branch contains predicted methionine transporters MetT, preceded by either candidate S-boxes (in Gram-positive bacteria) or MetJ binding sites (in γ-proteobacteria). The third branch includes predicted tyrosine transporters TyrT, most of which are members of the tyrosine T-box regulon (59). The fourth branch includes orthologs of malate : lactate antiporter MleN from *B.subtilis* (60). Thus analysis of candidate regulatory signals allows us to tentatively assign specificities to the remaining three large sub-groups of transporters from the NhaC superfamily.

Analysis of the methionine-specific regulatory signals allowed us to identify two more hypothetical methionine-related ABC transport systems in Gram-positive bacteria. The first one, named *mtsABC*, is present in all streptococci, some lactobacilli, and *C.perfringens*, *S.aureus* and *B.cereus* (Table 1). MtsA (SMU.1935c in *Streptococcus mutans*) has five predicted transmembrane segments and is not similar to any known protein. The MtsB and MtsC components (SMU.1934c and SMU.1933c) are similar to typical ATP-binding (CbiO) and transmembrane (CbiQ) components of various ABC transporters, respectively. In most cases *mtsABC* genes are clustered with the *hcp* gene (*SMU.1936c*) that encodes a hypothetical cytosolic protein.

The second methionine-related ABC transport system, named *tom* (for Transporter for Oligopeptides or Methionine), belongs to a large family of oligopeptide ABC transporters. In *B.cereus* and *Listeria monocytogenes*, the *tom* gene clusters (*BC0207-BC0208-BC0209-BC0210-BC0211* and *LMO2196-LMO2195-LMO2194-LMO2193-LMO2192*, respectively) are preceded by S-boxes. Moreover, *E.faecalis* has a single *tomA* gene (*EF3081*) for a substrate-binding component of transporter, which is preceded by a methionine-specific T-box. The available data were insufficient to assign specificities of these two methionine-regulated transporters by the genome context analysis and metabolic reconstruction. One possibility is that they are involved in the uptake of some methionine precursors or oligopeptides.

Methionine salvage and SAM recycling. The methionine salvage genes *mtnKSUVWXYZ* involved in the methylthioribose utilization in *B.subtlis* (21–23) were identified only in three *Bacillus* species, always as members of the S-box regulon (Table 1). In *B.cereus*, one S-box motif was found upstream of the *BC0768-767-766* gene cluster (named *mtnABC*) encoding a hypothetical ABC transport system. An ortholog of this system exists in the genome of *B.stearothermophilus*, but not in other studied bacterial genomes. Based on S-box regulatory site, similarity to the ribose ABC transporter and phylogenetic co-occurrence with the *mtnKSUSVWXYZ* genes, we tentatively assign the methylthioribose specificity to the MtnABC

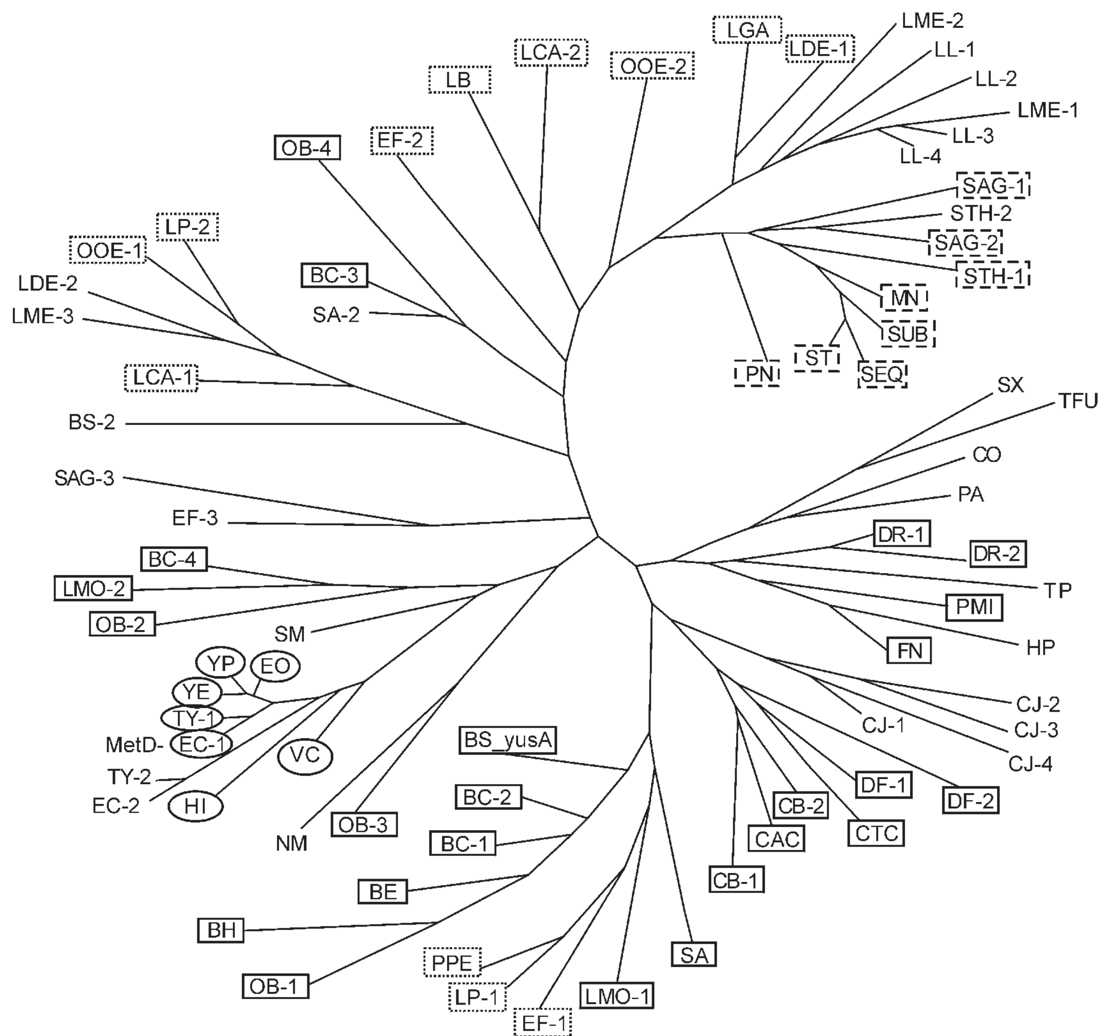

Figure 3. Phylogenetic tree of the substrate-binding components MetQ of bacterial methionine transporters from the MUT family. Proteins are denoted by genome abbreviations (listed in Table 1). Multiple paralogs are numbered. Genes predicted to be regulated by S-boxes, T-boxes and MET-boxes are boxed by solid, dotted and broken lines, respectively. Genes known and predicted to be regulated by the methionine repressor MetJ (data not shown) are circled. Additional genome abbreviations are: *Escherichia coli* (EC), *Salmonella typhi* (TY), *Yersinia pestis* (YP); *Yersinia enterocolitica* (YE); *Erwinia carotovora* (EO), *Vibrio cholerae* (VC), *Haemophilus influenzae* (HI), *Fusobacterium nucleatum* (FN), *Helicobacter pylori* (HP), *Deinococcus radiodurans* (DR), *Treponema pallidum* (TP), *Caulobacter crescentus* (CO) and *Pseudomonas aeruginosa* (PA).

transport system. The absence of MtnABC in *B.subtilis*, which also can grow on methylthioribose (2,19), suggests existence of other specific transport systems.

In addition to the autotrophic pathway of cysteine biosynthesis, some bacterial species (including *B.subtilis*) can synthesize this amino acid through the reverse transsulfuration pathway using methionine as a precursor (61). In this pathway, methionine is first converted to homocysteine via the SAM recycling pathway (Figure 1). In an attempt to discover missing genes of this pathway, we started with positional analysis of the *B.subtilis yrhA* and *yrhB* genes apparently encoding the cystathionine β-synthase and γ-lyase, respectively (12). Orthologs of these genes were found in all *Bacillus* species, *S.aureus* and three clostridia and are always clustered forming one candidate operon (Table 1). Moreover, the *yrhAB* genes form possible operons with the SAH nucleosidase gene *mtn* in bacilli, and with orthologs of the *B.subtilis luxS* gene in *O.iheyensis*, *C.perfringens* and *Clostridium botulinum*. The functional role of the *B.subtilis* LuxS protein is not known, though its structure has been recently determined (62). The autoinducer-2 production protein LuxS from proteobacteria catalyzes transformation of *S*-ribosylhomocysteine to homocysteine (63). We assign the previously missing ribosylhomocysteinase function of the SAM recycling pathway to the *luxS* gene product. Orthologs of the *luxS* and *mtn* genes were identified in most Gram-positive bacteria, corroborating the existence of SAM recycling pathways in these organisms.

Two other genes co-localized in positional clusters with the *yrhAB* genes (*yrrT* from bacilli and *ubiG* from clostridia) both contain SAM-binding motifs and are similar to various SAM-dependent methyltranferases (Table 1). It was known that SAH is synthesized from SAM as a by-product of numerous methylation reactions in the cell (2). Based on co-localization with the *yrhAB* genes, we propose that the pathway of reverse synthesis of cysteine from methionine

could require specific SAM-dependent methylases, and assign this role to YrrT in bacilli and UbiG in clostridia.

The hypothesis that the *ubiG-yrhAB* operon of *C.acetobutylicum* is involved in the cysteine synthesis is further supported by observation of a cysteine-specific T-box upstream of this operon, likely mediating repression by cysteine (12). Moreover, a backward-directed S-box motif located immediately downstream of this operon could regulate formation of an antisense transcript for this locus, assuming activation of the *ubiG-yrhAB* operon by methionine. Thus we predict that genes for the reverse transsulfuration pathway in *C.acetobutylicum* are apparently expressed only in the conditions of methionine excess and cysteine deficiency (see Figures 1 and 5). Some other mode of regulation by interference of transcription from the complementary strand also could be involved, but it would not change the main conclusion.

Two highly similar genes of unknown function, *yxjH* and *yxjG*, are members of the S-box regulon in *B.subtilis* (24). They encode proteins with moderate similarity to the C-terminal part of the B_{12}-independent methionine synthase MetE. Mutation analysis has showed that these genes are not required for the *de novo* methionine synthesis in *B.subtlis* (12). Orthologs of these genes (denoted *yxjH*) were identified in various Gram-positive bacteria, several proteobacteria and archaea (see the phylogenetic tree in Figure 6). Similar to *B.subtilis*, the *yxjH* genes in *O.iheyensis* and *L.monocytogenes* are S-box regulated. Moreover, most *yxjH* orthologs from Lactobacillales and *Streptococcus* species belong to the methionine T-box and MET-box regulons, respectively (Table 1).

Negative regulation of the B_{12}-independent methionine synthase isozymes by B12-element and vitamin B_{12} is common in bacteria possessing both B_{12}-dependent and B_{12}-independent isozymes (34). At that, vitamin B_{12}-responsive regulatory elements were detected upstream of the *yxjH* orthologs in *Rhodopseudomonas palustris* and *Bacteroides fragilis*, two bacteria possessing B_{12}-dependent methionine synthases (34). It indicates that these two B_{12}-regulated *yxjH* orthologs likely function as B_{12}-independent methionine synthases.

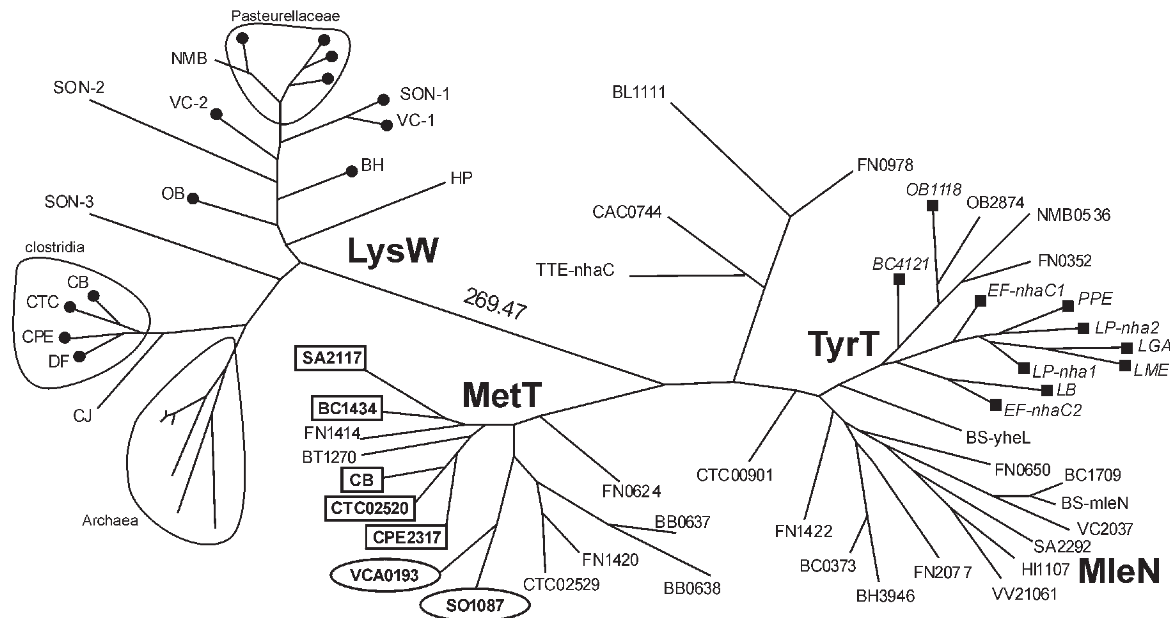

Figure 4. Phylogenetic tree of the NhaC Na^+:H^+ antiporter superfamily including predicted methionine-, lysine- and tyrosine-specific transporters. Gene identifiers are shown for annotated complete genome sequences. Genes predicted to be regulated by the methionine-specific S-box motif and MetJ repressor are boxed and circled, respectively. Genes predicted to be regulated by *LYS* elements (29) are denoted by filled circles. Genes predicted to be regulated by tyrosine-specific T-boxes (48) are denoted by black squares.

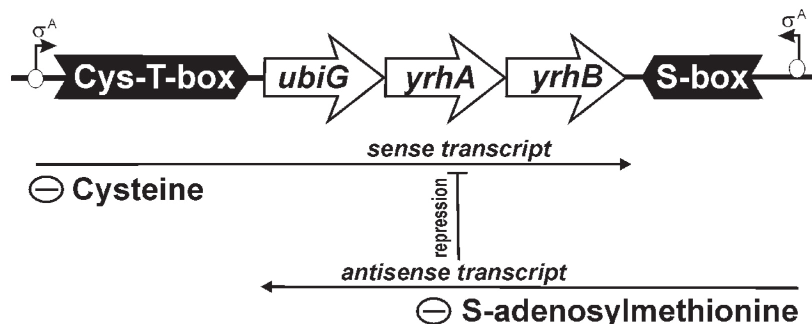

Figure 5. Predicted regulation of the *C.acetobutylicum ubiG-yrhBA* operon by *S*-adenosylmethionine-specific S-box and cysteine-specific T-box regulatory signals.

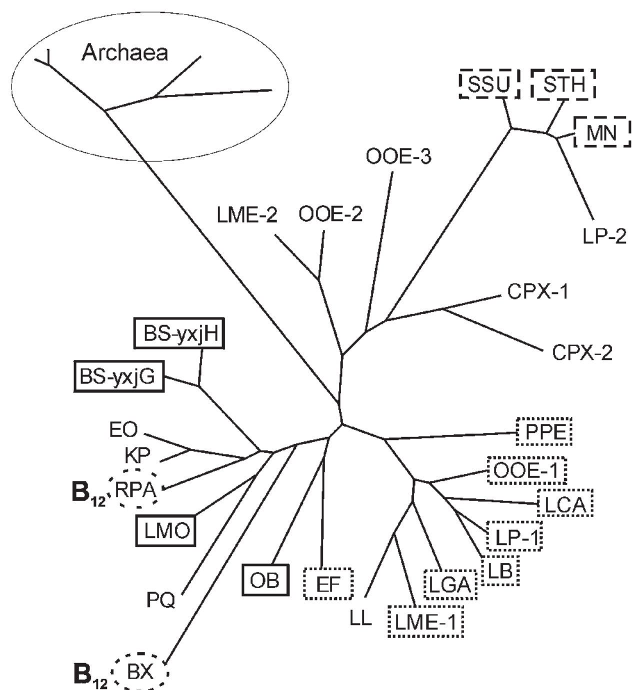

Figure 6. Phylogenetic tree of bacterial orthologs of the *B.subtilis yxjH* gene. Proteins are denoted by the genome abbreviations (listed in Table 1). Multiple gene paralogs are numbered. Genes predicted to be regulated by S-boxes, T-boxes and MET-boxes are boxed by solid, dotted and broken lines, respectively. Genes predicted to be regulated by the vitamin B_{12}-specific RNA regulatory element (28) are circled by broken lines. Additional genome abbreviations are: *Erwinia carotovora* (EO), *Klebsiella pneumoniae* (KP), *Rhodopseudomonas palustris* (RPA), *Mannheimia haemolytica* (PQ), *B.fragilis* (BX) and *Chlamydophila pneumoniae* (CPX).

Further, based on results of positional analysis and phylogenetic profiling, we suggest that YxjH could function as an alternative methionine synthase enzyme mainly involved in the SAM recycling pathway. Indeed, we identified several cases of co-localization of the *yxjH* genes with the candidate SAM recycling gene *luxS* (in *Lactobacillus gasseri*, *Lactobacillus casei* and *Leuconostoc mesenteroides*) and with the methionine biosynthesis genes *metICB* (in *Oenococcus oeni*). Notably, the latter genome lacks both methionine synthase genes (*metE* and *metH*). In addition, we found methionine-regulated *yxjH* genes in some species from the order Lactobacillales, which lack genes for the *de novo* methionine synthesis but possess genes for both methionine transport and SAM recycling. However, our assignment of the methionine synthase role to YxjH contradicts the observation that the *metE B.subtilis* mutant is a methionine auxotroph, thus suggesting that MetE is the only methionine synthase in this species (12,64). Additional experiments are required to prove this tentative assignment of *yxjH* to the SAM recycling pathway.

Other candidate members of methionine regulons. Candidate methionine transporter operons in *Streptococcus*, *Oenococcus*, *Leuconostoc* and *Oceanobacillus* species include a hypothetical gene (*hmrA* or *hmrB*) from the M20 family of zinc metallopeptidases. Known members of this family catalyze the release of an N-terminal amino acid, usually neutral or hydrophobic, from a polypeptide (65). Single *hmrA* gene of *B.cereus* (*BC3176*) is a member of the S-box regulon. In addition, methionine regulons of *B.cereus*, *S.aureus*, *L.mesenteroides* and three *Streptococcus* species contain a putative metal-dependent hydrolase gene, named *mdh* (genes *BC0395*, *SA0343* and *SMU1172* in complete genomes and COG entry 1878). In other Gram-positive bacteria, the *mdh* genes are not regulated by methionine, at least not by known systems. The role of the *hmrA*, *hmrB* and *mdh* gene products in the methionine metabolism is not clear.

In *S.coelicolor* and *T.fusca*, S-box precedes the *SCD95A.26* gene encoding a hypothetical pyridoxal-phosphate dependent enzyme (PFAM entry PF00291). Enzymes of this class catalyze various reactions in the metabolism of amino acids (66). *SCD95A.26* is a distant homolog of the threonine synthase gene *thrC*. However, actinobacteria have a proper *thrC* gene located within the threonine biosynthetic gene cluster. Since the complete genome of *S.coelicolor* lacks orthologs of known genes for the synthesis of homocysteine, the direct precursor of methionine, we tentatively suggest that *SCD95A.26* could be involved in the homocysteine synthesis in actinobacteria.

DISCUSSION

Methionine biosynthetic and transport genes are regulated by different mechanisms in various microbial species (Figure 7). The S-box system in Bacillales and Clostridia orders and the methionine-specific T-box system in Lactobacillales are RNA-dependent regulatory systems that both control transcription termination, although in a different manner. The S-box RNA structure is stabilized by direct binding of an effector molecule, *S*-adenosylmethionine, whereas the T-box senses lack of amino acid via the presence of uncharged tRNAs. Bacteria from the Streptococcaceae family, lacking both these RNA-dependent systems, are predicted to have a classical DNA-dependent system for regulation of the methionine metabolism, which includes numerous MET-box sites and the MtaR regulatory protein. Regulation of the methionine biosynthesis and transport genes in Gram-negative enterobacterium *E.coli* is also DNA-dependent and involves two known transcription factors, activator MetR and repressor MetJ, that bind operators absolutely different from MET-box (8). The most intriguing mode of regulation was found for the *C.acetobutylicum ubiG-yrhAB* operon, which seems to be regulated by both S- and T-box systems (Figure 5).

Inspite of a variety of regulatory systems, the cores of the methionine regulons in Gram-negative and Gram-positive species almost completely coincide. They include most genes required for the *de novo* methionine synthesis and transport. In contrast, SAM synthase gene *metK* belongs to the methionine regulons only in bacilli/clostridia and γ-proteobacteria, via the S-box and MetJ regulatory systems, respectively. In lactobacilli and streptococci this gene is not regulated by either the T-box or MET-box systems. One possible explanation for this could be the use of different effector molecules for the methionine regulons in these taxonomic groups. SAM is a known effector of the S-box and MetJ regulatory systems, whereas methionine itself is involved in the regulation by the T-box system via methionine-specific tRNA. Apparently,

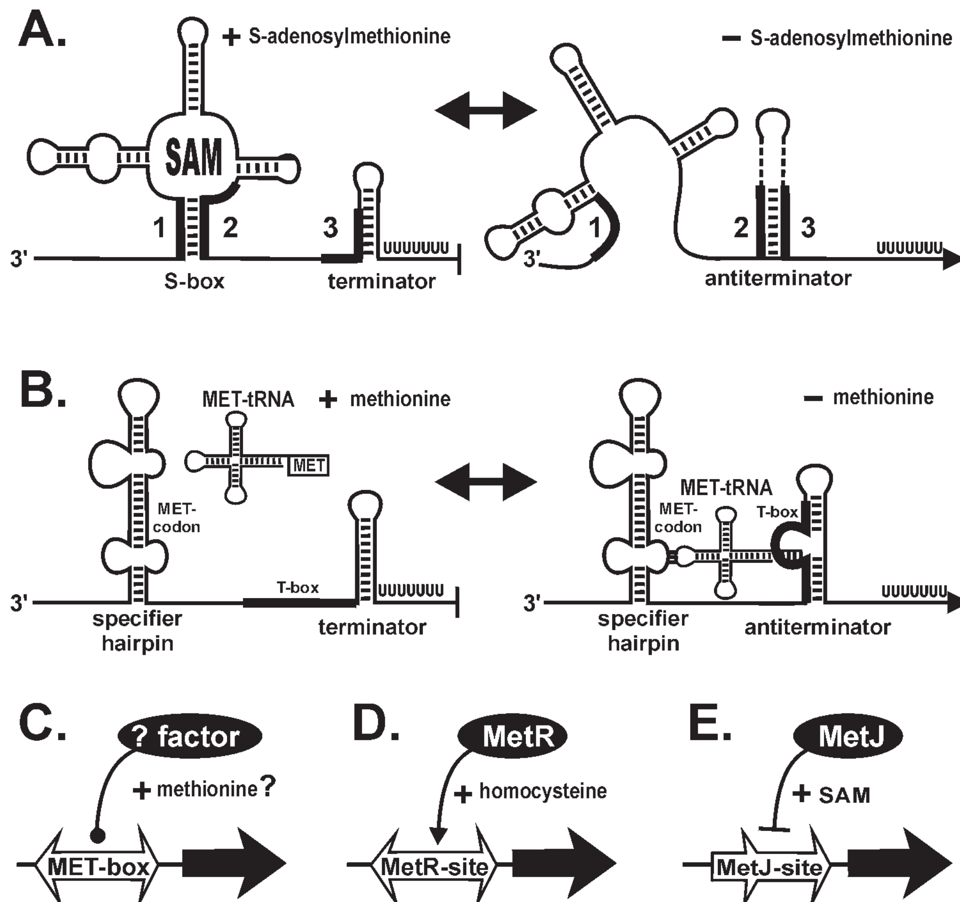

Figure 7. Regulation of the methionine biosynthesis and transport genes in bacteria by S-box riboswitch (**A**), T-box structure (**B**), unknown transcriptional regulator binding the MET-box operator (**C**), activator MetR (**D**) and repressor MetJ (**E**). The mechanisms of the S-box and T-box regulation involve the formation of alternative RNA structures. SAM stabilizes the repressing conformation (blunt end) in (A), whereas in (B) the anti-repressing conformation (arrow) folds in the presence of uncharged methionyl-tRNA. The regions involved in alternative interactions are shown by bold lines or curves. Poly-U tracts in terminators are shown above the 'RNA line'. Large arrows in (C–E) denote regulated genes or operons.

in the latter case it is not necessary to regulate SAM synthesis by the methionine availability. The effector molecule for the predicted MET-box system in streptococci is not known. In this bacterial group, MET-boxes precede most of the methionine biosynthesis and transport genes but not the *metK* genes (Table 1). Using the same biochemical logic we tentatively assign the role of the regulatory molecule in streptococci to methionine itself.

Compared to other riboswitches, S-boxes demonstrate somewhat mosaic phylogenetic distribution. They were observed in two major groups of firmicutes, bacilli and clostridia, and in a number of other taxons. Although riboswitches are subject to frequent horizontal transfer (32), and it cannot be ruled out in this case, especially for isolated proteobacterial genomes (*Xanthomonas* and *Geobacter*), the existence of S-boxes in a variety of genomes argues for their ancient origin. In particular, the most parsimonious evolutionary scenario for firmicutes seems to be the following. S-boxes were present in the last common ancestor of bacilli and clostridia, i.e. the last common ancestor of all firmicutes, and it was lost in the Streptococcaceae and Lactobacillales lineages. In streptococci, the role of S-boxes in the regulation of methionine metabolism was assumed by the transcriptional regulator MtaR with the MET-box binding signal, whereas in lactobacilli, the S-box regulon was absorbed by the expanded Met-T-box regulon, which initially included only aminoacyl-tRNA synthetases. One possible example of an early stage of such expansion could be the Met-T-box upstream of the *metICFE-mdh* operon in *S.aureus*. On the other hand, degradation of a S-box is exemplified by the case of *cysH* genes of *B.subtilis* and closely related genomes.

The comparative analysis of regulation supplemented by genome context and similarity search techniques allowed us to identify several new methionine-related transport systems, assign missing functions of the SAM recycling pathway, and demonstrate variability in the upper part of the methionine pathway in different species. Furthermore, we identified genome-specific extensions of the methionine regulon that involve genes shared with other biochemical pathways. This study not only demonstrates once again the power of comparative genomics in functional annotation of genes, prediction of regulatory interactions and metabolic reconstruction, but also provides one of the first examples where the possible scenario of evolution of several systems regulating one metabolic pathway could be tentatively reconstructed.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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