In Vivo Analysis of Various Substrates Utilized by Cystathionine γ-Synthase and *O*-Acetylhomoserine Sulfhydrylase in Methionine Biosynthesis

Y. Hacham, U. Gophna,† and R. Amir**

*Plant Science Laboratory, Migal Galilee Technology Center, Kiryat-Shmona, Israel; and †Department of Molecular Microbiology and Biotechnology, Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv, Israel

To gain insight into the evolution of the methionine biosynthesis pathway, in vivo complementation tests were performed. The substrate specificity of three enzymes that intrinsically use different homoserine-esterified substrates and have different sulfur assimilation pathways was examined: two cystathionine γ-synthases (the *Escherichia coli* enzyme that naturally utilizes *O*-succinylhomoserine [OSH]) and the *Arabidopsis thaliana* enzyme that naturally exploits *O*-phosphohomoserine [OPH]. Both of these act through the transsulfuration pathway. The third enzyme investigated was *O*-acetylhomoserine (OAH) sulfhydrylase of *Leptospira meyeri*, representing the enzyme that utilizes OAH and operates through the direct sulfhydrylation pathway. All the three enzymes were able to utilize OSH and OAH as substrates, with different degrees of efficiency, but only the plant enzyme was able to utilize OPH as a substrate. In addition to their inherent activity in the transsulfuration pathway, the two cystathionine γ-synthases were also capable of acting in the direct sulfhydrylation pathway. Based on the phylogenic tree and the results of the complementation tests, we suggest that the ancestral gene was able to act as OAH or OSH sulfhydrylase. In some bacteria and plants, this ancient enzyme most probably evolved into a cystathionine γ-synthase, thereby maintaining the ability to utilize various homoserine-esterified substrates, as well as various sulfur sources, and thus keeping the multisubstrate specificity of its ancestor. In some organisms, this ancestral gene probably underwent a duplication event, which resulted in a cystathionine γ-synthase and a separate OAH or OSH sulfhydrylase. This led to the development of two parallel pathways of methionine biosynthesis, transsulfuration and direct sulfhydrylation, in these organisms. Although both pathways exist in several organisms, most seem to favor a single specific pathway for methionine biosynthesis in vivo.

Introduction

The methionine biosynthetic pathways of bacteria, yeast, and plants are identical up to homoserine and from homocysteine to methionine (fig. 1). However, from homoserine to homocysteine, the pathways differ in a number of aspects: the precursor for homoserine esterification, the method by which the sulfur atom assimilates into the carbon skeleton of methionine, the branch point between the methionine and threonine pathways, the intermediate metabolites, and the enzymes that react in these biosynthetic routes (fig. 1). One major variation is the homoserine esterification reaction. In gram-negative facultative aerobic and anaerobic bacteria, homoserine is acylated to *O*-succinylhomoserine (OSH) in the presence of succinylcoenzyme A (fig. 1*A*). This reaction is catalyzed by homoserine *O*-succinyltransferase, the *Met*A gene product (Saint-Girons et al. 1988; Old et al. 1991). In gram-positive bacteria of the genera *Bacillus*, *Corynebacterium*, and *Brevibacterium*, as well as in yeast and fungi, homoserine is acetylated to *O*-acetylhomoserine (OAH) in the presence of acetylcoenzyme A, catalyzed by homoserine *O*-acetyltransferase (Ozaki and Shiio 1982; Langin et al. 1986) (fig. 1*B*). In plants, homoserine is esterified with a phosphoryl group to produce *O*-phosphohomoserine (OPH), in a reaction catalyzed by homoserine kinase (fig. 1*C*) (Macnitol et al. 1981).

Another major difference in the methionine biosynthesis pathways involves sulfur atom assimilation into the methionine backbone. There are two alternative subroutes. The first is the transsulfuration pathway, in which cysteine is the sulfur donor and is incorporated into the homoserine ester to form cystathionine. This reaction is catalyzed by cystathionine γ-synthase (CGS), an enzyme present in plants and in most species of bacteria. This is followed by β-elimination, which converts cystathionine into homocysteine, in a reaction catalyzed by cystathionine β-lyase (fig. 1*A* and *C*). The net effect of these two reactions is the transfer of the thiol group of cysteine, a three-carbon compound, to form the four-carbon compound, homocysteine. Hence, this pathway is called transsulfurylation. In the second subroute, the sulfur donor is sulfide, which is incorporated into the homoserine ester by OAH-sulfhydrylase or OSH-sulfhydrylase to form homocysteine (fig. 1*B*). This route bypasses the transsulfuration reaction and is therefore called direct sulfhydrylation (Yamagata 1989). The latter pathway is found, for example, in *Saccharomyces cerevisiae* (Kerjan, Cherest, and Surdin-Kerjan 1986), *Leptospira meyeri* (Belfaiza et al. 1998), *Brevibacterium flavum* (Ozaki and Shiio 1982), and *Pseudomonas aeruginosa* (Foglino et al. 1995). In some bacteria, both of these subroutes of sulfur atom assimilation may occur, as shown by in vitro studies (Kanzaki et al. 1986).

The methionine biosynthesis pathways differ also at the point where threonine and methionine biosynthesis pathways branch. In plants, this branch point occurs at OPH, which is used as a substrate for both threonine and cystathionine biosynthesis (fig. 1*C*). In bacteria and fungi, these pathways diverge from homoserine (one metabolite upstream of OPH). In these latter groups, OPH is used solely for threonine biosynthesis (fig. 1*A* and *B*).

To elucidate the evolutionary processes, which led to different types of methionine biosynthesis in various organisms, we analyzed the substrate specificity of three enzymes that utilize different homoserine-esterified

Key words: amino acid, methionine biosynthesis pathway, cystathionine γ-synthase, *O*-acetylhomoserine sulfhydrylase, substrate specificity, evolution.

E-mail: rachel@migal.org.il.

Mol. Biol. Evol. 20(9):1513–1520. 2003
DOI: 10.1093/molbev/msg169
Molecular Biology and Evolution, Vol. 20, No. 9,
© Society for Molecular Biology and Evolution 2003; all rights reserved.

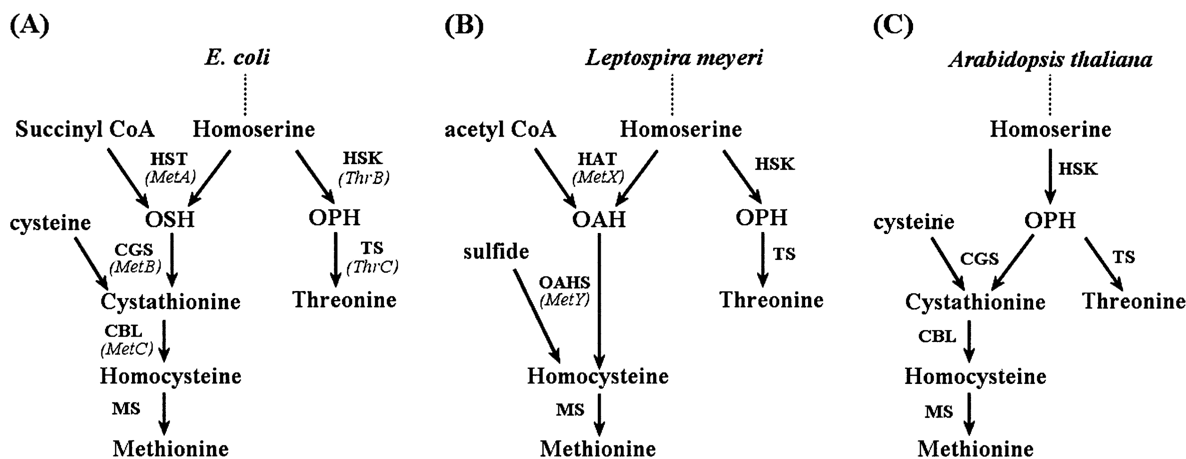

FIG. 1.—Methionine and threonine biosynthesis pathways in *Escherichia coli* (*A*), *Leptospira meyeri* (*B*), and *Arabidopsis thaliana* (*C*). The pathways are identical up to homoserine. The relevant genes involved in methionine and threonine synthesis in *E. coli* and in *L. meyeri* are indicated (*A* and *B*). Abbreviations: OAH = *O*-acetylhomoserine; OSH = *O*-succinylhomoserine; OPH = *O*-phosphohomoserine; HSK = homoserine kinase; HAT = homoserine *O*-acetyltransferase; OAHS = *O*-acetylhomoserine sulfhydrylase; HST = homoserine *O*-succinyltransferase; TS = threonine synthase; CGS = cystathionine γ-synthase; CBL = cystathionine β-lyase; MS = methionine synthase.

substrates in vivo. We analyzed two CGSs, the first from *Escherichia coli* that utilizes OSH and the second from *Arabidopsis thaliana* that utilizes OPH. Both of them take part in the transsulfuration pathway. The third enzyme studied was OAH-sulfhydrylase of *Leptospira meyeri*, representing the enzyme that utilizes OAH and acts through direct sulfhydrylation. It was found that these three enzymes could utilize OAH and OSH in vivo, although at different levels of efficiency, but only the plant enzyme could utilize OPH. In addition, the two CGSs were revealed to be bifunctional enzymes, which can participate in the direct sulfhydrylation pathway, as well as in their inherent transsulfuration pathway.

Materials and Methods

Materials

O-succinyl-L-homoserine was purchased from Sigma. *O*-acetyl-L-homoserine was synthesized from L-homoserine, based on a previously reported method (Nagai and Flavin 1971).

Bacterial Strains

The bacteria strains used in this study are listed in table 1. The double and triple mutations of *E. coli* were prepared by P1 transduction (Miller 1972). To prepare a double mutant of *met*B-*thr*B (YBB1), the *thr*B gene was transferred by P1 transduction from Hfr 3000 YA73, after lysogenizing the latter with CAG12093 (*car*B96:Tn10) (the *car* gene is closest to the threonine operon in the *E. coli* genome). This enabled the use of tetracycline selection for subsequent *thr*B:Tn10 transductions. The *thr*B:Tn10 was introduced into LE392 to form the YBB1 mutant. The nature of the YBB1 cells was tested on minimal medium, lacking threonine and methionine.

The double mutant of *met*A-*thr*C (YAC1) was prepared after P1 lysogenized the GIF41 strain (*thr*C). The *thr*C:Tn10 was introduced into the AB1932 strain to form the YAC1 mutant. The triple mutant of *met*A-*met*B-*thr*B (YABB1) was prepared as described above; that is, the original strain was GT1140 (*met*A-*met*B) lysogenized with *thr*B:Tn10, through P1 transduction.

Two different media were used to grow the *E. coli* bacterial strains. The first was Luria-Bertani (LB), and the second was the M9 minimal medium (Sambrook, Fritsch, and Maniatis 1989). The latter medium was supplemented with 1 mg/ml thiamine and 40 mg/ml of each amino acid, with the exception of threonine and methionine. For positive control, methionine and threonine were added at a concentration similar to the other amino acids. The antibiotics used were ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; and tetracycline, 12.5 μg/ml.

Plasmid Construction

For the complementation tests, three different genes, the *Met*B gene of *E. coli*, the *At*CGS gene of *A. thaliana*, and the *Met*Y gene of *L. meyeri*, were subcloned into the plasmid, pACYC184 (Cm^r Tet^r) at *Sph*I and *Sal*I sites, canceling the tetracycline resistance of this plasmid. However, the use of these sites enabled the alien genes to be controlled by the promoter of the tetracycline resistance gene. The forward primers contained the Shine-Delgarno sequences, derived from the expression vector pQE (Qiagen), which also included the *Sph*I restriction site, containing the ATG translation-initiation codon. A *Sal*I site was inserted into the reverse primer.

Arabidopsis CGS cDNA was PCR amplified from a flower cDNA library, kindly donated by the Arabidopsis Biological Resource Center (Columbus, Ohio). Fragments of DNA, encoding mature CGS (without its plastid transit peptide), starting with Val-68 (Ravanel et al. 1998), were amplified with the following primers: 5′-AGCATGCAAAGAGGAGAACTATGGT CCGTCAGCTGAGCATTAAAGCC-3′ and 5′-ATCAGATGGCTTCGAGAG CTTGAAGAA-3′. *Met*B was amplified from *E. coli* DNA with the primers: 5′-AAAAGAGGAGAACTATGACGCGTAAACAGGCCACC-3′ and 5′-ATTACCCCTTGTTTGCAGC CCGG-3′. The *Met*Y of *L. meyeri* was amplified from the

Table 1
***E. coli* Strains Used in This Study**

E. coli Strain	Genotype	Origin or Reference	Source
Hfr 3000 YA73	*Thr*B1000, *rel*A1, *spo*T1, *thi*-1	J. Bacteriol (1974) 117:133	I. Saint-Girons
CAG12093	*Car*B96::Tn10, *rph*-1, l-	Microbiol. Rev. (1989) 53:1	*E. coli* Genetic Stock Center
AB1932	*Lac*Y1, *tsx*6, *gin*V44, *gal*K2, *xyl*A7, *arg*H1, *thi*-1, *met*A28	Genetics (1966) 53:1119	*E. coli* Genetic Stock Center
GT1107	*Met*C:Tet, *met*B1, *lac*-3, *gal*K2, *gal*T22, *sup*E44	J. Bacteriol. (1998) 180: 250	I. Saint-Girons
LE392	*Met*B1, *hsdR*514, *supE*44, *supF*58, *lacY*1, *tryp*R55, *gal*K2, *gal*T22		Stratagene, USA
Gif41	*Thr*C *1001*, *thi*-1, *rel*A, *spo*T1	J. Bacteriol. (1974) 117: 133	I. Saint-Girons
GT1140	*Met*A::Cmr, *met*B1, *lac*-3, *gal*K2, *gal*T22, *sup*E44, *hsd*R	J. Bacteriol. (1998) 180: 250	I. Saint-Girons
GUC41	*Met*C, *exb*B, *thr*, *leu*, *ton*A	J. Bacteriol. (1973) 114: 1225	*E. coli* Genetic Stock Center
YBB1	As LE392 with *thr*B	Transduction of LE392 by a P1 lystae grown on *car*B96::Tn10	This study
YAC1	As AB1932 with *thr*C	Transduction of AB1932 by a P1 lystae grown on *car*B96::Tn10	This study
YABB1	As GT1140 with *thr*B	Transduction of GT1140 by a P1 lystae grown on *car*B96::Tn10	This study

pb12 plasmid, kindly donated by Isabel Saint-Girons, using the following primers: 5′-AGCATGCAAAGAGGA-GAACT ATGGTAGGACCATCGGGGGAATC-3′ and 5′-ATCAGATATTTTTTAATG CCTCTTC-3′. The amplified fragments were ligated to a pGEMT-cloning vector (Promega), and subsequently into pACYC184, via *Sph*I/*Sal*I sites. The nucleotide sequences of the constructed plasmids were verified by DNA sequencing. *Met*X of *L. meyeri* on pET20b+ plasmid was kindly provided by Isabel Saint-Girons. This plasmid possesses gene resistance to ampicillin (Bourhy et al. 1997).

Complementation of *E. coli* Methionine Auxotrophs

pACYC-184 carrying the three different genes was employed to transform the various *E. coli* mutants. The transformed bacteria were plated on LB medium and grown overnight at 37°C. Colonies were picked and grown in LB broth to an optical density OD_{600} of 1.0. One milliliter of the bacteria was pelleted and washed twice with M9 medium containing 0.2% glucose. The bacteria were plated on an M9 plate or grown in M9 liquid medium.

Sequence Analysis

Protein sequences of the organisms listed in table 2 were aligned, using ClustalX (Thompson et al. 1997), a Windows application based on ClustalW (Higgins et al. 1996). Phylogenetic trees were reconstructed by quartet-puzzling maximum likelihood using Tree-Puzzle, version 5.0 (Strimmer and von Haeseler 1996), with 10,000 puzzling steps, applying quartet sampling for substitution process and neighbor-joining tree for rate variation determination. Trees were visualized using the program TreeView (Page 1996).

Results

The Ability of the Enzymes to Use OSH as a Substrate for Methionine Synthesis

The ability of the enzymes *A. thaliana* CGS and *L. meyeri* OAH-sulfhydrylase to use OSH for methionine synthesis instead of their natural substrates, OPH and OAH, respectively, was tested. The recombinant genes encoding each of these enzymes were employed separately to complement the double mutant, *met*B-*thr*B (YBB1). In this mutant, OPH cannot be synthesized and OSH cannot be utilized. Consequently, OSH accumulates as the sole substrate of homoserine esterification for the utilization by these enzymes. The *E. coli* CGS gene was used as a positive control, and the unmodified plasmid vector was employed as a negative control (*Met*B and pACYC, respectively, in fig. 2*A*). The *Arabidopsis* enzyme enabled 93% bacterial mutant growth, as compared with that obtained by the *Met*B product of *E. coli* (fig. 2*B*). This indicates that the plant enzyme can efficiently utilize OSH, even though this substrate is apparently not present in plant cells.

No complementation and growth were obtained when the *Met*Y gene of *L. meyeri* was used. However, previous studies have demonstrated that the *Met*Y gene can complement the *met*B mutant of *E. coli*, albeit with a low efficiency (Belfaiza et al. 1998). We found, as described below, that *Met*Y can partially complement the GT1107 mutant, which lacks the transsulfuration pathway, causing it to accumulate OSH. GT1107, and *met*B mutants also produce OPH, but OAH-sulfhydrylase apparently does not use OPH, because it did not complement the YAC1 mutant that produced only OPH (as described below). This suggests that OAH-sulfhydrylase of *L. meyeri* can utilize OSH, although at low efficiency.

The Ability of the Enzymes to Use OAH as a Substrate

The ability of the *A. thaliana* and *E. coli* CGSs to use OAH was tested by complementing the triple mutant *met*A, *met*B, and *thr*B (YABB1) with the genes encoding each of these enzymes separately. In this mutant, neither OPH nor OSH could be produced, and the mutant also lacked the *E. coli* CGS, which may utilize OAH as a substrate. OAH that was synthetically synthesized was added to M9 liquid medium (40 mg/ml). OAH-sulfhydrylase of *L. meyeri* was used as a positive control. As

Table 2
Protein Sequences Used in This Study

Number	Organism	Enzyme	Gene	Length (Amino Acids)	Accession Number
		CGS			
1	*Salmonella enterica*		*Met*B	386	NP_457953
2	*Escherichia coli*		*Met*B	386	AAB03071
3	*Xanthomonas axonopodis*		*Met*B	405	AAM37884
4	*Neisseria meningitidis* MC58		NMB0802	385	NP_273844
5	*Corynebacterium glutamicum* 1		*Met*B	386	T47234
6	*Helicobacter pylori*		*Met*B	380	NP_222819
7	*Bacillus subtilis* 1		*yrh*B	379	A69974
8	*Bacillus subtilis* 2		*yjc*I	373	A69847
9	*Mycobacterium tuberculosis* 1		*Met*B	388	NP_215595
10	*Solanum tuberosum*		*CGS*	539	AAF74981
11	*Fragaria vesca*		*CGS*	545	CAA04772
12	*Arabidopsis thaliana*		*CGS*1	563	BAA24699
13	Glycine max		*cys*	536	AAD34548
14	*Zea mays*		*CGS*1	509	AAB61348
15	*Nicotiana tabacum*		*Met*B	445	AAD16143
16	*Oryza sativa*		*CGS*1	360	AAG38873
		OSH Sulfhydrylase			
17	*Pseudomonas aeruginosa*		*Met*Z	403	F83256
18	*Neisseria meningitidis* MC582		NMB1609	389	NP_274615
19	*Rhizobium etli*		*Met*Z	394	CAA09983
20	*Mycobacterium tuberculosis* 2		MT0402	406	AAK44624
		OAH Sulfhydrylase			
22	*Corynebacterium glutamicum* 2		*Cg*l0653	437	BAB98046
23	*Leptospira meyeri*		*Met*Y	442	T44655
24	*Emericella nidulans*		*cys*D	437	T45481
25	*Neurospora crassa*		B13I18.90	454	CAB99179
26	*Saccharomyces cerevisiae*		*Met*17	444	NP_013406
27	*Candida albicans*		*Met*15	440	AAF01453
28	*Schizosaccharomyces pombe*		SPBC428.11	429	T40463

shown in figure 3, although the control plasmid vector did not support mutant growth, the plant and the bacterial CGSs efficiently complemented this mutant, demonstrating that they are able to utilize OAH.

The ability of *E. coli*-CGS to utilize OAH was also examined in an *E. coli met*A mutant, which had been transformed with the *L. meyeri Met*X gene. The *met*A mutant is a methionine auxotroph, since it can neither produce OSH, nor can it utilize OPH (Michaeli and Ron 1981). The *Met*X gene encodes for homoserine *O*-acetyltransferase that combines homoserine and acetyl-coenzyme A to form OAH (Bourhy et al. 1997). Thus, complementation of the *E. coli met*A mutant by the *L. meyeri Met*X gene would mean that the endogenous *E. coli* CGS can utilize OAH. The results obtained from an experiment in which bacteria were incubated for 72 h in M9 liquid medium show significant growth, although it was low (reflected by an OD_{600} of 0.47), which further indicated that CGS of *E. coli* can utilize OAH.

The Ability of the Enzymes to Use OPH as a Substrate

OPH serves as a substrate for plant CGS enzymes (fig. 1*C*). Although OPH exists in bacteria in the threonine biosynthesis pathway, the *E. coli* CGS cannot utilize it for methionine biosynthesis in vivo, as deduced from the methionine auxotrophy of the *met*A mutant (Michaeli and Ron 1981). To ascertain whether the OAH-sulfhydrylase encoded by the *Met*Y gene can use OPH as a substrate, the *thr*C-*met*A (YAC1) double mutant was formed. In this mutant, OSH was not produced, and OPH was expected to accumulate, since it could not be converted into threonine because of a mutation in the *thr*C gene. The YAC1 mutant was transformed with the recombinant *Met*Y gene, as well as with the *Arabidopsis* CGS gene, as a positive control. Whereas the *At*CGS complemented this mutant, the *Met*Y gene did not (fig. 4*A* and *B*). This implies that only the plant enzyme can use OPH for methionine synthesis.

The Ability of the CGS Enzymes to Act in the Direct Sulfhydrylation Pathway

To test whether CGSs can act through direct sulfhydrylation and use sulfide in vivo, the *Arabidopsis AtCGS* and *E. coli Met*B genes were used to transform GT1107. This *E. coli* mutant lacks the transsulfuration pathway, because of double mutations in *met*B and *met*C. The *Met*Y gene of *L. meyeri* was used as a positive control, since the enzyme encoded by this gene uses sulfide and acts through direct sulfhydrylation (fig. 1*B*). It was found that *Met*Y alone could partially complement GT1107, because its encoded enzyme could utilize OSH as a substrate for direct sulfhydrylation (fig. 5). However, in transformants of *Met*X and *Met*Y, complementation was further improved (fig. 5), as shown by the fact that the natural substrate of the *Met*Y product, OAH, was produced.

As shown in figure 5, *Met*B of *E. coli* could complement the GT1107 mutant. Moreover, the efficiency

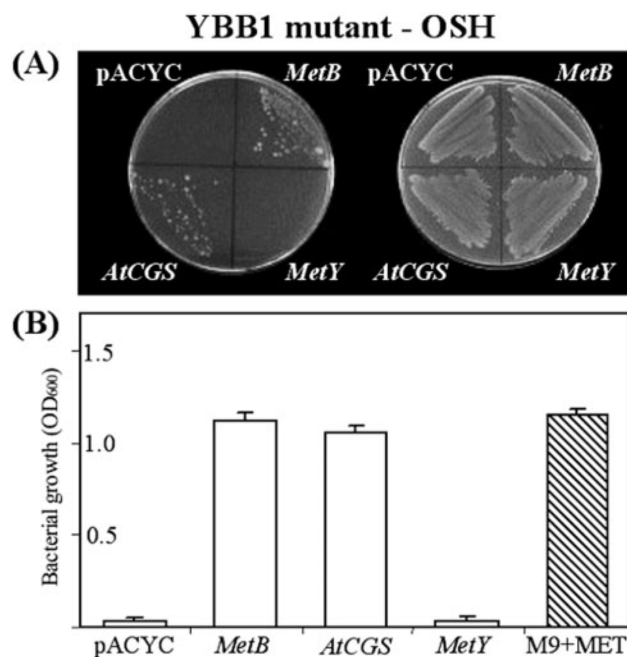

FIG. 2.—Functional complementation (*A*) and bacterial growth (*B*) of *E. coli* mutants YBB1 that produce *O*-succinylhomoserine as a sole substrate for CGS and *O*-acetylhomoserine sulfhydrylase. The mutants were transformed by the plasmid pACYC184 (negative control) or with this plasmid containing *Met*B of *E. coli* (positive control), *At*CGS of *Arabidopsis thaliana* or *Met*Y of *Leptospira meryi*. The transformed bacteria were plated onto M9 minimal medium, with methionine (40 μg/ml) (*A*, right panel) or without methionine (*A*, left panel). The optical density was measured after 72 h of growth in M9 liquid medium (unshaded bars), whereas the results from the M9 medium supplemented with methionine (shaded bar) were recorded after 24 hours. All the transformed bacteria began to grow at an OD_{600} of 0.03. The data are represented as the mean ± SD of three individual repeats.

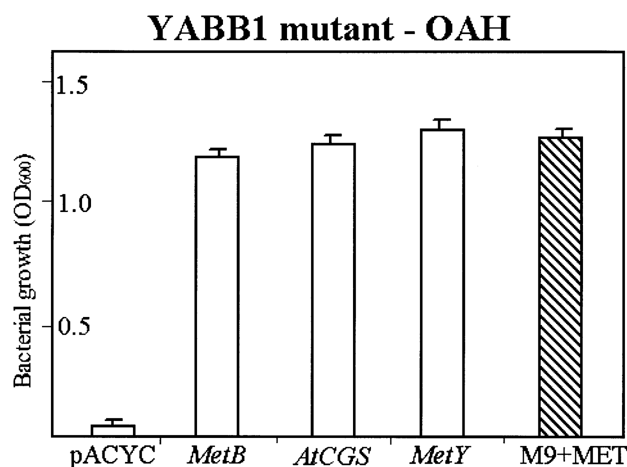

FIG. 3.—The bacterial growth of *E. coli* mutants, YABB1, transformed by the plasmid pACYC184 (negative control) or with this plasmid containing *Met*Y of *Leptospira meryi* (positive control), *Met*B of *E. coli*, or *At*CGS of *Arabidopsis thaliana*. The transformed bacteria grew with *O*-acetylhomoserine (40 μg/ml) (unshaded bars) in the M9 minimal medium. The optical density was measured after 72 h, whereas the results from the M9 medium supplemented with methionine (shaded bar) were recorded after 24 hours. All the transformed bacteria began to grow at OD_{600} of 0.03. The data are represented as the mean ± SD of three individual repeats.

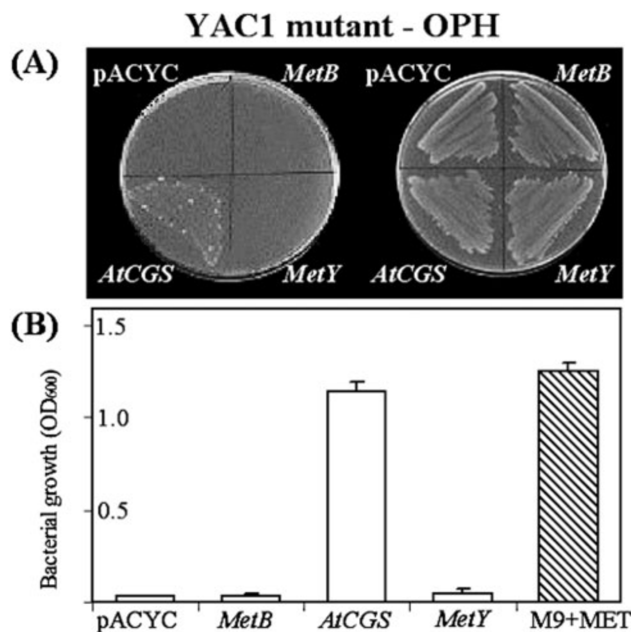

FIG. 4.—Functional complementation (*A*) and bacterial growth of *E. coli* mutants YAC1 that produce *O*-phosphohomoserine as a sole substrate for CGS and *O*-acetylhomoserine sulfhydrylase (*B*). The mutants were transformed by the plasmid pACYC184 (negative control) or with this plasmid containing *At*CGS of *Arabidopsis thaliana* (positive control), *Met*B of *E. coli*, or *Met*Y of *Leptospira meryi*. The transformed bacteria were plated on M9 minimal medium with methionine (*A*, right panel) or without methionine (40 μg/ml) (*A*, left panel). The optical density was measured after 72 h of growth in M9 liquid medium (unshaded bars), whereas the results from the M9 medium supplemented with methionine (shaded bar) were recorded after 24 hours. All the transformed bacteria began to grow at the OD_{600} of 0.03. The data are represented as the mean ± SD of three individual repeats.

of this complementation was comparable to that obtained when *Met*B was used to complement the YBB1 mutant (cf. fig. 5 with fig. 2*B*). This implies that the *E. coli* CGS can act through direct sulfhydrylation as efficiently as through transsulfuration. The *Arabidopsis At*CGS enzyme could also complement GT1107 (fig. 5) but less efficiently compared with those obtained when this gene was used to

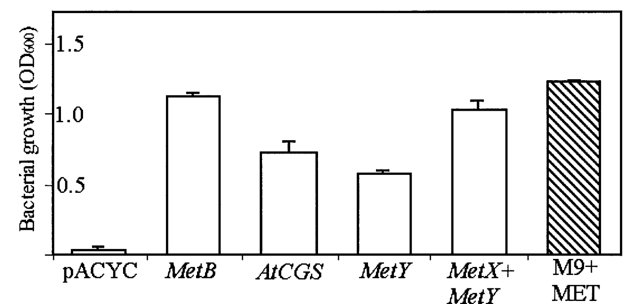

FIG. 5.—Growth of *E. coli* mutants GT1107 lacking the trans-sulfuration pathway, transformed with the plasmid pACYC184 (negative control) or with this plasmid containing *Met*B gene of *E. coli*, *At*CGS gene of *Arabidopsis thaliana* or *Met*Y, *Met*X of *Leptospira meyeri*. The measurements were recorded after 72 h of growth in M9 medium (unshaded bars), whereas the results of the M9 medium supplemented with methionine were taken after 24 hours (shaded bar). All the transformed bacteria began to grow at OD_{600} of 0.03. The data are represented as the mean ± SD of three individual repeats.

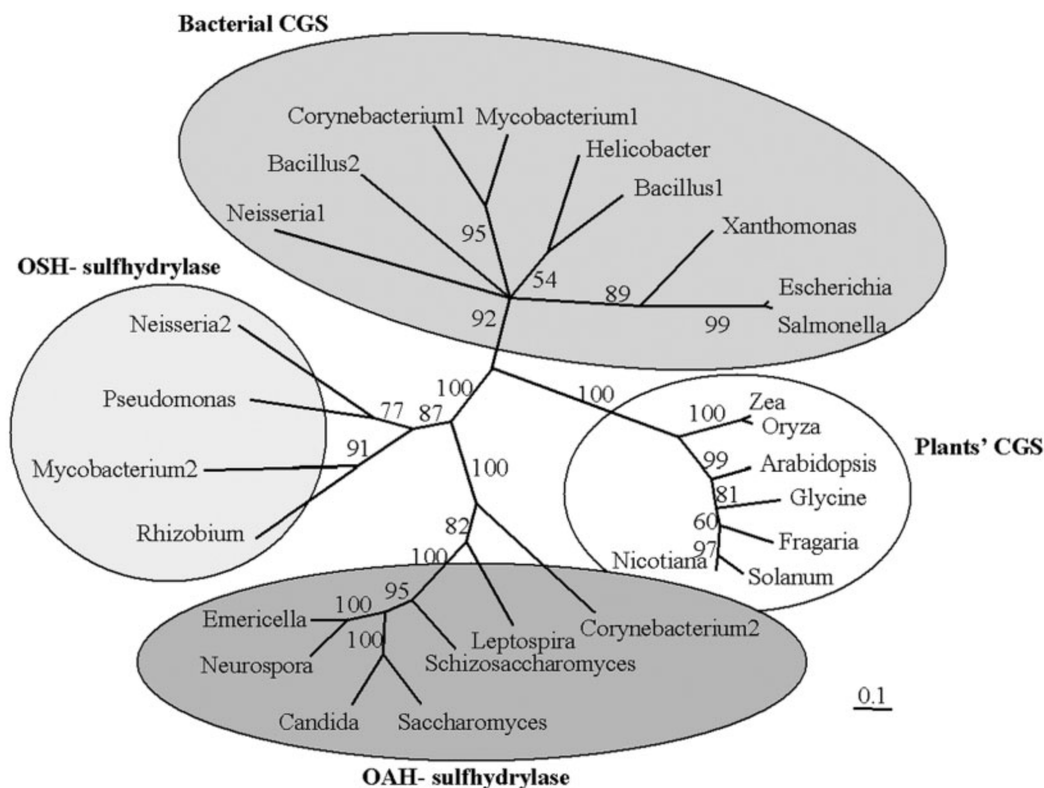

FIG. 6.—A phylogenic tree of various CGSs, *O*-acetylhomoserine sulfhydrylase, and *O*-succinylhomoserine sulfhydrylase from bacteria, fungi, and plants. Proteins sequences and abbreviations used are described in table 2. The tree was generated by the quartet-puzzling software Tree-Puzzle, using 10,000 puzzling steps. Numbers denote quartet puzzling support values for internal branches (in percent). Scale bar represents number of substitutions per site.

complement YAC1 through the transsulfuration pathway (cf. fig. 5 with fig. 4*B*).

Evolution of the Transsulfuration and the Direct Sulfhydrylation Pathways

To learn more about the evolutionary history of the methionine biosynthesis pathway, a phylogenic tree of amino acid sequences of CGS, OAH, or OSH sulfhydrylase enzymes of various bacteria, fungi, and plants was constructed, using quartet-puzzling maximum likelihood. The same tree topology was also observed when the neighbor-joining method was applied for tree reconstruction.

Four major groups could be distinguished with high internal branch support values (fig. 6). The first group consisted of CGSs of plants that naturally utilize OPH and cysteine and act through transsulfuration. The second group contained CGSs of bacteria that intrinsically use OSH (*Escherichia* or *Salmonella*) or OAH (*Bacillus*, *Helicobacter*, or *Corynobacterium*) through transsulfuration. The third group contained enzymes of bacteria that are active in the direct sulfhydration pathway and use OSH as a substrate. The fourth group included enzymes of bacteria and fungi that function through direct sulfhydration but utilize OAH. These two latter groups appear to share a common ancestor, which most probably functioned though direct sulfhydration.

Bacteria such as *Mycobacterium*, *Corynobacterium*, and *Neisseria* have two different enzymes for methionine biosynthesis, a CGS that functions through transsulfuration and an OAH or an OSH sulfhydrylase that acts through direct sulfhydration. These two different enzymes cluster in different branches of the phylogenic tree (fig. 6).

Discussion

The results of the present study provide evidence that, although various organisms evolved to produce only one of the homoserine-esterified substrates, their enzymes retain the ability to use other homoserine-esterified substrates as well. The *Arabidopsis* CGS, for example, can use the three homoserine esterified substrates with similar efficiency but in nature produces only OPH. The ability of the enzymes to use different homoserine-esterified substrates was also shown by Kanzaki et al. (1986), who screened 75 bacterial species in vitro and discovered that the majority of the CGS bacterial enzymes can use both OSH and OAH. Theoretically, all of these homoserine-esterified substrates can be produced in bacterial, fungal, and plant cells because their precursors, homoserine, acetylcoenzyme A, and succinylcoenzyme A, are available. It is likely that cells of different organisms favor one of these homoserine-esterified substrates, due to additional factors, which may depend on the metabolic network of the species and its ecological niche.

Although, in general, gram-negative bacteria produce OSH, whereas gram-positive bacteria produce OAH, this

division is not unambiguous. The results obtained from two different species of *Pseudomonas* demonstrated, for example, that they use different homoserine-esterified substrates: *P. syringae* produces OAH and *P. aeruginosa* produces OSH, although they belong to the same genus (Andersen, Beattie, and Lindow 1998). Phylogenic analysis (fig. 6) could not discriminate between bacterial CGS homologs that use OAH (*Bacillus* and *Helicobacter*) and those that utilize OSH (*Salmonella* and *E. coli*). However, the two branches of direct sulfhydrylation clustered according to the different homoserine-esterified substrates, OAH or OSH (fig. 6). The plant CGS homologs that utilize OPH were grouped in a specific cluster, which is distant from the other branches of the tree. This may reflect its substrate specificity (OPH) and the fact that the plants enzymes possess N-terminal regulatory region that is absent from bacterial and fungal enzymes (Hacham, Avraham, and Amir 2002).

The ability of the enzymes to use different homoserine-esterified substrates was accompanied by their ability to utilize different sulfur sources. CGS enzymes of *Arabidopsis* and *E. coli*, which can utilize several homoserine-esterified substrates, can also substitute cysteine for sulfide as a sulfur source in methionine synthesis and thus acts also as sulfhydrylase. In vitro tests with bacterial and plant enzymes have shown that they can use various sulfur and thiol compounds for methionine synthesis (Kanzaki et al. 1987; Ravanel et al. 1998).

Although the CGS enzymes have the ability to act through transsulfuration as well as through direct sulfhydrylation, recently available genome sequence data indicate that some bacteria and fungi possess separate independent loci for these two different routes. Thus, both CGS and OAH or OSH sulfhydrylase are found in these organisms. This has been described, for example, in *Niesseria meningitidis* and *Corynebacterium glutamicum* (Hwang et al. 1999), in *Mycobacterium tuberculosis*, and in the yeast *S. cervicia* (Marzluf 1997). However, it is not clear whether these two different pathways are physiologically active or whether they are involved in methionine synthesis in these organisms. Genetic and biochemical evidence shows that in *C. glutamicum*, the transsulfuration and the direct sulfhydrylation pathways are utilized with almost equal efficiency (Hwang et al. 2002). However, in yeast, it was shown that direct sulfhydrylation is the active route (Marzluf 1997). Complementation tests and activity measurements demonstrated that *P. aeruginosa* and *P. putida* are capable of synthesizing methionine by OSH or OAH sulfhydrylase and CGS activities, although the direct sulfhydrylation pathway is strongly favored (Foglino et al. 1995; Vermeij and Kertesz 1999). In both bacteria, the transsulfuration pathway is highly expressed when cysteine is supplied as the sole sulfur source (Vermeij and Kertesz 1999).

The direct sulfhydrylation pathway probably exists in all species of plant, bacteria, and fungi. In some bacteria and fungi, only OAH or OSH sulfhydrylase are present. In other organisms, sulfhydrylase activity is performed by CGS (i.e., CGS has the ability to act also as a sulfhydrylase). This is the case, for example, in plants (Ravanel et al. 1998), in *B. subtilis* (Auger et al. 2002), and in *E. coli* (Simon and Hong 1983). The *E. coli* enzyme can act through transsulfuration and direct sulfhydrylation with nearly the same efficiency, as was demonstrated in this study. In many other bacteria and fungi, the two pathways are separate and the organisms show both CGS and sulfhydrylase activities, performed by independent enzymes.

Based on the phylogenic tree and the results obtained from the complementation tests, we conclude that the ancestral gene encoded an enzyme that had the OAH /OSH sulfhydrylase activities. It is likely that early organisms relied exclusively on the sulfhydrylase activity and that the ancestral protein evolved by gradual mutations, which resulted in increasingly efficient utilization of one of the homoserine-esterified substrates. Thus, although one homoserine-esterified substrate was preferred, the alternative source could still be utilized, albeit less efficiently. In other organisms, the ancestral gene evolved to form the CGS. Although CGS uses cysteine as a sulfur source for homocysteine synthesis, it is also able to utilize sulfide and to act as sulfhydrylase. Thus, the CGS maintains the substrate flexibility of its progenitor. In some cases, the CGS underwent duplication, as suggested for *B. subtilis*. Two CGS paralogs were found in this bacterium, although the function of one of them (yrhB) remains unclear (Auger et al. 2002). In some bacteria and fungi, which have both CGS and an independent sulfhydrylase enzyme, the ancestral gene had probably been duplicated, and subsequent mutations gradually produced the activities of the present-day enzymes. The duplication is likely to have occurred early in evolution, since these two paralogs (CGS and OAH or OSH sulfhydrylase) now cluster in different branches of the phylogenic tree (fig. 6). The plant CGS homologs may have develop from the bacterial CGS, but their homoserine esterified substrate changed to OPH, although they can still use the other homoserine-esterified substrates as well as sulfide. Nonetheless, direct sulfydrylation apparently works at low efficiency in plants, as shown for *Arbidopsis* in this study, and by a tobacco mutant that lacks the cystathionine β-lyase (i.e., lacks the transsulfuration pathway). The latter mutant is auxotrophic to either methionine or homocysteine (Negrutiu et al. 1985).

The evolutionary rationale behind the different organisms' choice of various routes for methionine synthesis, including the sulfur sources and the homoserine-esterified substrates, is probably linked to their interior metabolic network. This may be dependent on their natural habitat specialization, and further study is warranted to clarify this point.

Acknowledgments

We are very grateful to Isabel Saint-Girons for providing some of the bacterial mutants and the genes of *L. meyeri*. We would also like to thank Gadi Galili for his critical reading of the manuscript and Einat Canaan for her technical assistance. This research was supported by Grant No. 410/98-2 of the Israel Science Foundation.

Literature Cited

Andersen, G., G. Beattie, and S. Lindow. 1998. Molecular characterization and sequence of a methionine biosynthetic locus from *Pseudomonas syringae*. J. Bacteriol. **180**:4497–4507.

Auger, S., W. H. Yuen, A. Danchin, and I. Martin-Verstraete. 2002. The metIC operon involved in methionine biosynthesis in *Bacillus subtilis* is controlled by transcription antitermination. Microbiology **148**:507–518.

Belfaiza, J., A. Martel, D. Margarita, and I. Saint-Girons. 1998. Direct sulfhydrylation for methionine biosynthesis in *Leptospira meyeri*. J. Bacteriol. **180**:250–255.

Bourhy, P., A. Martel, D. Margarita, I. Saint-Girons, and J. Belfaiza. 1997. Homoserine *O*-acetyltransferase, involved in *Leptospira meyeri* methionine biosynthetic pathway, is not feedback inhibited. J. Bacteriol. **179**:4396–4398.

Foglino, M., F. Borne, M. Bally, G. Ball, and J. C. Patte. 1995. A direct sulfhydrylation pathway is used for methionine biosynthesis in *Pseudomonas aeruginosa*. Microbiology **141**: 431–439.

Hacham, Y., T. Avraham, and R. Amir. 2002. The N-terminal region of arabidopsis cystathionine gamma synthase plays an important role in methionine metabolism. Plant Physiol. **128**:454–462

Higgins, D. G., J. D. Thompson, and T. J. Gibson. 1996. Using Clustal for multiple sequence alignments. Methods Enzymol. **266**:383–402.

Hwang, B. J., Y. Kim, H. B. Kim, H. J. Hwang, J. H. Kim, and H. S. Lee. 1999. Analysis of *Corynebacterium glutamicum* methionine biosynthetic pathway: isolation and analysis of metB encoding cystathionine gamma-synthase. Mol. Cells **9**:300–308.

Hwang, B. J., H. J. Yeom, Y. Kim, and H. S. Lee. 2002. *Corynebacterium glutamicum* utilizes both transsulfuration and direct sulfhydrylation pathways for methionine biosynthesis. J. Bacteriol. **184**:1277–1286

Kanzaki, H., M. Kobayashi, T. Nagasawa, and H. M. Yamada. 1986. Distribution of the two kinds of cystationine gamma-synthase in various bacteria. FEMS Microbiol. Lett. **33**:65–68.

———. 1987. Purification and characterization of cystathionine gamma synthase type II from *Bacillus sphaericus*. Eur. J. Biochem. **16**:105–112.

Kerjan, P., H. Cherest, and Y. Surdin-Kerjan. 1986. Nucleotide sequence of the *Saccharomyces cerevisiae MET25* gene. Nucleic. Acids Res. **14**:7861–7871.

Langin, T., G. Faugeron, C. Goyon, A. Nicolas, and J. I. G. Rossignol. 1986. The *Met2* gene of *Saccharomyces cervisiae*: molecular cloning and nucleotide sequence. Gene **49**:283–293.

Macnitol, P. K., A. H. Datko, J. Giovanelli, and H. Mudd. 1981. Homocysteine biosynthesis in green plants: physiological importance of the transsulfuration pathway in *Lemna paucicostata*. Plant Physiol. **68**:619–625.

Marzluf, G. A. 1997. Molecular genetics of sulfur assimilation in filamentous fungi and yeast. Ann. Rev. Microbiol. **51**:73–96.

Michaeli, S., and E. Z. Ron. 1981. Construction and physical mapping of plasmids containing the *metA* gene of *Escherichia coli* K-12. Mol. Gen. Genet. **182**:349–354.

Miller, J. H. 1972. Use of P1 transduction from pools of colonies to obtain transpositions near a gene of interest. Pp. 357–364 *in* A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Nagai, S., and M. Flavin. 1971. Synthesis of *O*-acetylhomoserine. Methods Enzymol. **17B**:423–424.

Negrutiu, I., D. De Brouwer, R. Dirks, and M. Jacobs. 1985. Amino acid auxotrophs from protoplast cultures of *Nicotiana plumbagiifolia*, Viviani. Mol. Gen. Genet. **199**:330–337.

Old, I., S. Phillips, P. Stockley, and I. Saint-Girons. 1991. Regulation methionine biosynthesis in the enterobacteriaceae. Prog. Biophys. Mol. Biol. **56**:145–185.

Ozaki, H., and I. Shiio. 1982. Methionine biosynthesis in *Brevibacterium flavum*: properties and essential role of *O*-acetyl-homoserine sulfhydrylase. J. Biochem. **91**:1163–1171.

Page, R.D. 1996. TreeView: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12**: 357–358.

Ravanel, S., B. Gakiere, D. Job, and R. Douce. 1998. Cystathionine gama-synthase from *Arabidopsis thaliana*: purification and biochemical characterization of the recombinant enzyme overexpressed in *Escherichia coli*. Biochem. J. **331**:639–648.

Saint-Girons, I., C. Parsot, M. M. Zakin, O. Barzu, and G. Cohen. 1988. Methionine biosynthesis in enterobacteriaceae: biochemical, regulatory, and evolutionary aspects. CRC Crit. Rev. Biochem. **23**(Suppl. 1):S1–S42.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Simon, M., and J. Hong. 1983. Direct homocysteine biosynthesis from *O*-succinylhomoserine in *Escherichia coli*: an alternate pathway that bypass cystathionine. J. Bacteriol. **153**: 558–561.

Strimmer, K., and A. von Haeseler. 1996. Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. Mol. Biol. Evol. **13**:964–969.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25**:4876–4882

Vermeij, P., and M. A. Kertesz. 1999. Pathways of assimilative sulfur metabolism in *Pseudomonas putida*. J. Bacteriol. **181**: 5833–5837.

Yamagata, S. 1989. Role of *O*-acetyl-L-homoserine sulfhydrylase in microorganisms. Biochimie **71**:1125–1143.

Antony Dean, Associate Editor

Accepted April 30, 2003