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Identification of coenzyme M biosynthetic 2-phosphosulfolactate phosphatase

A member of a new class of Mg^{2+}-dependent acid phosphatases

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Coenzyme M (CoM; 2-mercaptoethanesulfonic acid) is the terminal methyl carrier in methanogenesis. Methanogenic archaea begin the production of this essential cofactor by sulfonating phosphoenolpyruvate to form 2-phospho-3-sulfolactate. After dephosphorylation, this precursor is oxidized, decarboxylated and then reductively thiolated to form CoM. A thermostable phosphosulfolactate phosphohydrolase (EC 3.1.3.–) catalyzing the second step in CoM biosynthesis, was identified in the hyperthermophilic euryarchaeon *Methanococcus jannaschii*. The predicted ORF MJ1140 in the genome of *M. jannaschii* encodes ComB, a Mg^{2+}-dependent acid phosphatase that is specific for 2-hydroxycarboxylic acid phosphate esters. Recombinantly expressed purified ComB efficiently hydrolyzes *rac*-2-phosphosulfolactate, (*S*)-2-phospholactate, phosphoglycolate and both enantiomers of 2-phosphomalate. In contrast to previously studied phosphoglycolate phosphatases, ComB has a low pH optimum for activity, a narrow substrate specificity and an amino acid sequence dissimilar to any biochemically characterized protein. Like other phosphatases that function via covalent phosphoenzyme intermediates, ComB can catalyze a transphosphorylation reaction. Homologs of *comB* are identified in all available cyanobacterial genome sequences and in genomes from phylogenetically diverse bacteria and archaea; most of these organisms lack homologs of other CoM biosynthetic genes. The broad and disparate distribution of *comB* homologs suggests that the gene has been recruited frequently into new metabolic pathways.

Keywords: acid phosphatase; coenzyme M; methanogenic cofactor; 2-phosphosulfolactate.

Coenzyme M (CoM; 2-mercaptoethanesulfonic acid) is the smallest known organic cofactor but its size belies its essential role in the methanogenic production of more than 400 million tons of methane per year [1]. Originally characterized as one of several coenzymes involved in the formation of methane by the archaeon *Methanobacterium* str. M.o.H [2], CoM has the distinction of being the terminal methyl carrier and a component of the key energy-yielding step that makes methanogenesis a viable physiology [3]. Because CoM is required for methanogenesis, its biosynthesis is an attractive target for developing inhibitors to control methane production by methanogens in anthropogenic sources.

Recently, CoM was identified as a cofactor in the oxidation of epoxyalkanes by the bacterium *Xanthobacter* str. Py2 [4]. In both methanogenesis and epoxyalkane oxidation, the thiol group of CoM forms a thioether bond with the alkyl substrate that is irreversibly broken by substitution to form a heterodisulfide bond with either coenzyme B (7-mercaptoheptanoylthreonine phosphate in methanogenesis) or a cysteine residue in protein component II (epoxyalkane oxidation). In both pathways this disulfide bond is reductively cleaved to regenerate CoM. The requirement for a reactive thiol group and an enzymatically recognizable sulfonate group complicates CoM formation, which probably requires seven enzymes for its complete biosynthesis (Fig. 1).

The proposed pathway for the biosynthesis of CoM from phosphoenolpyruvate (PEP) was deduced by analysis of labeling patterns of CoM purified from *Methanobacterium formicicum, Methanobrevibacter ruminantium*, and rumen isolate 10-16B grown on stable isotope-labeled acetates [5]. Incubation of PEP and bisulfite with cell extracts of *Methanobacterium formicium* produced sulfolactate, sulfopyruvate and sulfoacetaldehyde, establishing the role of these intermediates in the pathway [6]. Subsequently, two CoM biosynthetic enzymes have been identified in *Methanococcus jannaschii*: (*R*)-sulfolactate dehydrogenase (ComC) catalyzes the oxidation of (*R*)-sulfolactate (L-sulfolactate) [7] and sulfopyruvate decarboxylase (ComDE) subsequently decarboxylates sulfopyruvate to form sulfoacetaldehyde [8]. Because PEP, rather than pyruvate, is the specific precursor to CoM biosynthesis, the product of sulfite condensation must be phosphosulfolactate, requiring a phosphatase to generate sulfolactate. However phosphosulfolactate could not be detected by the analytical methods originally used to identify CoM biosynthetic intermediates [6].

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Abbreviations: CoM, coenzyme M; HAD, haloacid dehalogenase; IC_{50}, 50% inhibitory concentration; PEP, phosphoenolpyruvate; PGP, phosphoglycolate phosphatase; RubisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; TMS, trimethylsilyl.

Enzymes: phosphosulfolactate phosphohydrolase (EC 3.1.3.–); mitochondrial L-malate : NAD^+ oxidoreductase (EC 1.1.1.37).

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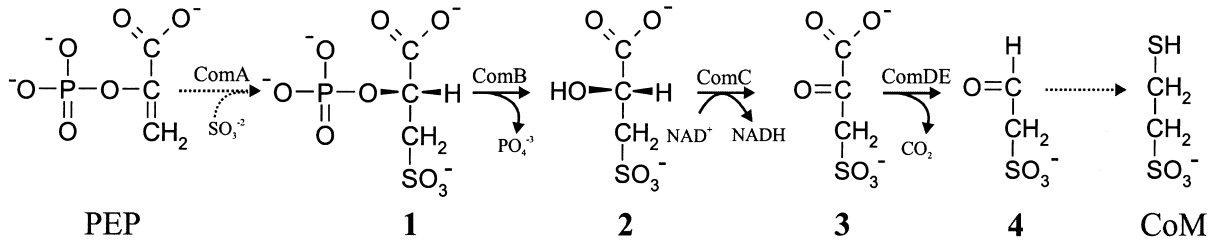

Fig. 1. Biosynthetic pathway of CoM. PEP is sulfonated to form phosphosulfolactate. Dephosphorylation by ComB phosphatase produces sulfolactate, which is oxidized to sulfopyruvate and then decarboxylated to sulfoacetaldehyde. Finally, reductive thiolation produces CoM. Reactions demonstrated *in vitro* using recombinant proteins are shown as solid arrows.

In this report we identify phosphosulfolactate phosphatase activity in *Methanosarcina thermophila and M. jannaschii* cell extracts and describe the 2-phospho-3-sulfolactate phosphohydrolase (EC 3.1.3.–; ComB) in *M. jannaschii*. We demonstrate that the product of *M. jannaschii* gene MJ1140 is a member of a new class of metal-dependent acid phosphatases that hydrolyzes the phosphate esters of 2-hydroxycarboxylic acids (Fig. 2). ComB specifically hydrolyzes (*S*)-phospholactate but not (*R*)-phospholactate; however, it hydrolyzes both enantiomers of 2-hydroxycarboxylic acids with pseudosymmetric centers of inversion such as (*S*)-phosphomalate and (*R*)-phosphomalate or (*R*)-phosphosulfolactate and (*S*)-phosphosulfolactate. To explain this unusual stereospecificity, we propose that ComB binds the substrate enantiomers in opposite orientations.

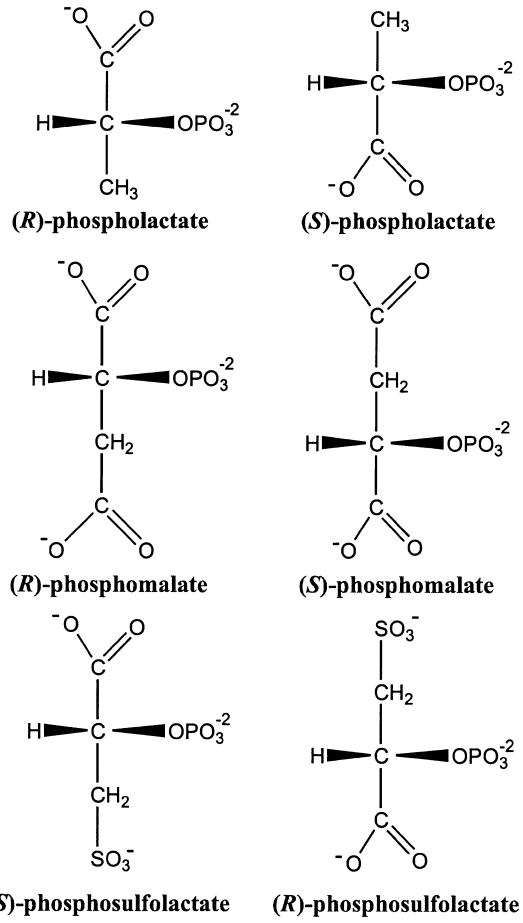

Fig. 2. Phosphate esters of 2-hydroxyacids. The phosphate ester of D-lactate is designated (*R*)-phospholactate.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma-Aldrich Corporation unless otherwise specified. (*RS*)-2-Phosphonooxy-3-sulfopropionic acid (*rac*-phosphosulfolactate), both enantiomers of 2-phosphonooxypropionic acid (phospholactate) and both enantiomers of 2-phosphonooxybutanedioic acid (phosphomalate) were synthesized chemically as described below. (*RS*)-3-Hydroxy-2-phosphonooxypropionic acid (*rac*-2-phosphoglycerate) was synthesized enzymatically using pyruvate kinase. Monoethyl phosphate was synthesized by reaction of ethanol with $POCl_3$, followed by hydrolysis to form the monoester [9]. (*R*)-Sulfolactate, which has the same relative configuration as (*S*)-lactate, was synthesized as described previously [7].

Preparation of trimethylsilyl derivatives

Trimethylsilyl (TMS) derivatives were generated by reacting 0.1–1-mg portions of dried samples with 50 μL of a 9 : 3 : 1 (v/v/v) mixture of pyridine, 1,1,1,3,3,3-hexamethyldisilazane and trimethylchlorosilane (Applied Science Laboratories) for 30 min at room temperature prior to gas chromatography [10]. GC-MS analyses were performed using a VG-70-70EHF GC-MS operating at 70 eV and equipped with a HP-5 column (0.32 mm × 30 m) programmed from 70 to 280 °C at 10 °C·min^{-1}. Under these conditions TMS derivatives of the respective compounds had the following retention times (min : s): lactate, 3 : 23; glycolate, 3 : 35; phosphate, 6 : 17; 2-phospholactate, 10 : 38 and phosphoglycolate 11 : 9.

Synthesis of *rac*-phosphosulfolactate

To a solution of trisodium PEP (23.4 mg, 0.1 mmol) dissolved in 50 μL water was added a solution of sodium bisulfite (100 mg, 1 mmol) in 180 μL water. After warming at 50 °C for 15 min the water was evaporated with a stream of nitrogen gas. For ^{1}H-NMR analysis the sample was dissolved in deuterated water. ^{1}H-NMR (400 MHz) analysis showed the complete absence of the PEP vinyl triplets at 5.383 and 5.197 p.p.m. and the appearance of three new

resonances from the products of the reaction which consisted of only phosphosulfolactate and pyruvate. ^{1}H-NMR peaks for phosphosulfolactate (in ^2H_2O) were δ 4.739 (1H, ddd, $J_{H\text{-}2\rightarrow P}$ = 6.9 Hz; $J_{H\text{-}2\rightarrow H\text{-}3}$ = 8.5 Hz; $J_{H\text{-}2\rightarrow H'\text{-}3}$ = 4.9 Hz, H-2) and 3.41 (2H, m, H-3 and H-3′); the resonance for pyruvate was δ 1.66 [s, H-3]. Assuming that no exchange of the pyruvate methyl occurred during the sample preparation, 29% of the PEP was hydrolyzed to pyruvate and the remaining PEP was converted into phosphosulfolactate.

The phosphosulfolactate solution was passed through a Dowex 50−8X H^+ (Bio-Rad) column (0.5 cm × 5 cm) to remove the sodium and decompose the bisulfite to SO_2 which was removed by evaporation with a stream of nitrogen. The resulting solution was adjusted to pH 7.3 with 1 M NaOH, placed on a DEAE Sephadex (Pharmacia) column (1 cm × 5 cm) and eluted with a linear gradient generated by mixing 200 mL water and 200 mL 2 M NH_4HCO_3. Fractions (8 mL) were collected and assayed for inorganic and organic phosphate. One peak for phosphate eluted maximally at fraction 5 and two equally intense peaks containing organic phosphate eluted maximally in fractions 11 and 14. The ^{1}H-NMR resonances of the organic phosphate-containing peaks differed slightly in their coupling patterns and line positions. However ^{1}H-NMR analysis of the combined peaks identified a single set of resonances. It was subsequently found that the chemical shifts and coupling patterns for the phosphosulfolactate were very dependent on the pH and ionic strength of the solution thus explaining the differences in the NMR of the two peaks. Treatment of the phosphosulfolactate present in each peak with bacterial alkaline phosphatase followed by ^{1}H-NMR of the sulfolactate product each gave the same ^{1}H-NMR (in ^2H_2O): δ 4.40 (1H, dd, $J_{H\text{-}2\rightarrow H\text{-}3}$ = 2.8 Hz; $J_{H\text{-}2\rightarrow H\text{-}3'}$ = 9.2 Hz, H-2); 3.35 (1H, dd, $J_{H\text{-}3\rightarrow H\text{-}2}$ = 2.8 Hz; $J_{H\text{-}3\rightarrow H\text{-}3'}$ = 15 Hz, H-3); 3.09 (1H, dd, $J_{H\text{-}3'\rightarrow H\text{-}2}$ = 9.2 Hz; $J_{H\text{-}3'\rightarrow H\text{-}3}$ = 15 Hz, H-3′). Material in fractions 11−14 was combined and lyophilized three times from water to recover the pure sample of the ammonium salt of *rac*-phosphosulfolactate used in these experiments.

Synthesis of (*R*)-phospholactate, (*S*)-phospholactate, (*R*)-phosphomalate and (*S*)-phosphomalate

To methyl (*R*)-(−)-lactate or methyl (*S*)-(+)-lactate (0.208 g, 2 mmol) dissolved in 1.3 mL of pyridine was added, with stirring at room temperature, diphenyl chlorophosphate (0.56 g, 2.09 mmol). After reaction overnight at room temperature 50 μL water was added and the pyridine was evaporated with a stream of nitrogen gas. The resulting sample was dissolved in 2 mL benzene and washed sequentially with 2 mL portions of water, 1 M HCl, water, and saturated $NaHCO_3$. After the solution was dried over Na_2SO_4 the benzene was evaporated with a stream of nitrogen gas to give a colorless oil of the methyl ester of lactyl diphenyl phosphate: M^+ = 336 *m/z* with important fragment ions at M^+-$COOCH_3$ = 277 *m/z*, and M^+-OC_6H_5 = *243 m/z* (italic number indicates base peak). ^{1}H-NMR (500 MHz) ($DCCl_3$): δ 7.16−7.36 (10H, m, phenyl), 5.096 (1H, dq, $J_{H\text{-}2\rightarrow P}$ = 8.5 Hz; $J_{H\text{-}2\rightarrow H\text{-}3}$ = 6.8 Hz H-2), 3.70 (3H, s, OCH_3), 1.52 (3H, d, $J_{H\text{-}3\rightarrow H\text{-}2}$ = 6.8 (Hz H-3). Hydrogenation of the sample dissolved in ethanol (100 mg·mL^{-1}, 10 mg PtO_2) for 1 h with 207 kPa hydrogen, gave the desired methyl ester of lactyl phosphate: ^{1}H-NMR (500 MHz) of the monotriethylamine salt ($DCCl_3$): δ 10.28 (1H, bs, HN), 4.80 (1H, dq, $J_{H\text{-}2\rightarrow P}$ = 8.9 Hz; $J_{H\text{-}2\rightarrow H\text{-}3}$ = 6.8 Hz, H-2), 3.71 (3H, s, OCH_3), 3.12 (6H, m, CH_2N), 1.495 (3H, d, $J_{H\text{-}3\rightarrow H\text{-}2}$ = 6.8 (Hz) 1.318 (8H, t, CH_3CH_2N). GC-MS analysis of the $(TMS)_2$ derivative of the product showed one GC peak having the desired mass spectra: M^+ = 328 *m/z* with important fragment ions at M^+-CH_3 = 313 *m/z* and M^+-$COOCH_3$ = 269 *m/z*. Samples were saponified by mixing the solution with 1 mL 1 M NaOH and evaporating the ethanol at 80 °C. Titration of the solutions with 1 M HCl to pH 7.0 was used to determine the phospholactate concentrations and to prepare them for incubations.

Both enantiomers of 2-phosphomalate were produced by the same procedure described for 2-phospholactate. (*R*)-phosphomalate was synthesized from dimethyl (*R*)-(+)-malate and (*S*)-phosphomalate was synthesized from dimethyl (*S*)-(−)-malate. The resulting colorless oil of the diphenyl dimethyl ester of phosphomalate showed M^+ = 394 *m/z* with fragment ions at M^+-31 = 363 *m/z*, and M^+-OC_6H_5 = 301 *m/z*. Hydrogenation of the samples dissolved in ethanol for 2 h with 207 kPa hydrogen gave the desired dimethyl ester of phosphomalate. After removal of the Pt by filtration, the samples were titrated with 1 M NaOH to pH 8.0 (phenolphthalein pink) to measure the moles of phosphate monoester deprotected. The carboxylic acid methyl esters were saponified overnight at room temperature after the addition of two equivalents of 1 M NaOH for each ester group. Titration of the solution with 1 M HCl to pH 7.0 was used to determine the moles of ester saponified. Both of these titrations showed that the desired reactions were quantitative. ^{1}H-NMR (500 MHz) of the resulting sodium salt (D_2O): δ 4.529 (1H, ddd, $J_{H\text{-}2\rightarrow P}$ = 4.95 Hz; $J_{H\text{-}2\rightarrow H\text{-}3}$ = 4.95 Hz; $J_{H\text{-}2\rightarrow H\text{-}3'}$ = 6.18 Hz, H-2), 2.754 (1H, dd, $J_{H\text{-}3\rightarrow H\text{-}2}$ = 4.95 Hz, $J_{H\text{-}3\rightarrow H\text{-}3'}$ = 15.25 Hz, H-3) and 2.661 (1H, dd, $J_{H\text{-}3'\rightarrow H\text{-}2}$ = 6.1 Hz; $J_{H\text{-}3'\rightarrow H\text{-}3}$ = 15.25 Hz, H-3′). Solution concentrations were established from the ratios of the signal intensities of the phosphomalate to the methyl resonances from added known amounts of (2,2,3,3−$^2H_2$)-3-(trimethylsilyl)-1-propanesulfonic acid and were confirmed by quantitation of inorganic phosphate released by alkaline phosphatase. Both phosphomalate enantiomers were purified by anion exchange chromatography on a Mono Q HR column (5 mm × 5 cm) (Pharmacia). Substrates were applied to the column in deionized water and then eluted using a 20-mL linear gradient to 1 M NH_4HCO_3. Phosphomalate was identified in 1-mL fractions containing 0.25−0.35 M NH_4HCO_3. NH_4HCO_3 was removed from samples by repeated drying under nitrogen gas and dissolution in deionized water.

Synthesis of [$^{18}O_5$]-(*S*)-phospholactate and analysis of its ^{18}O content

(*S*)-Phospholactate (14 μmol) was dissolved in 40 μL water (98 atom percentage ^{18}O), 3 μL 6 M HCl was added and the solution was heated at 100 °C for 15 min. After evaporation of the water with a stream of nitrogen gas the [$^{18}O_5$]-(*S*)-phospholactate was dissolved in 100 μL water. Analysis of the $(TMS)_3$ derivative of a portion of the sample by GC-MS showed, as measured from the M^+-15 ion at 371 *m/z*, that the product contained the following distribution of labeled

oxygen expressed as the atom percent excess of oxygen-18 over the natural abundance: 0.7 $^{18}O_0$, 1.0 $^{18}O_1$, 6.8 $^{18}O_2$, 25.1 $^{18}O_3$, 37.8 $^{18}O_4$, and 28.6 $^{18}O_5$. A rearrangement fragmentation of this molecule formed the $(TMS)_3$ derivative of the phosphate group which allowed the direct measurement of ^{18}O in the phosphate. From the M^+-15 = 299 *m/z* fragment of this ion the following the ^{18}O distribution was measured: 3.0 $^{18}O_0$, 13.2 $^{18}O_1$, 35.4 $^{18}O_2$, and 48.4 $^{18}O_3$. From the difference in the oxygen distribution in the total molecule and that measured in the phosphate group it was calculated that each of the carboxyl oxygens must be 77% labeled with ^{18}O. The sample contained only a small amount (< 1%) of inorganic phosphate that was found to have the following oxygen distribution: 48.1 $^{18}O_0$, 27.1 $^{18}O_1$, 15.4 $^{18}O_2$, 7.3 $^{18}O_3$, and 2.1 $^{18}O_4$.

Analysis of cleavage of phosphosulfolactate by *Methanosarcina thermophila* and *M. jannaschii*

Cell extract was prepared from *Methanosarcina thermophila* TM-1, a gift from J. G. Ferry, as described previously [11]. To 100 μL of cell extract (10 mg·mL^{-1} protein) was added 1.5 μmol *rac*-phosphosulfolactate under anaerobic conditions and the solution incubated at 50 °C for 2 h. Sulfolactate product was isolated from the incubation mixture and its presence confirmed by GC-MS analysis of the dimethyl ester O-methyl ether derivative [6]. *M. jannaschii* JAL-1 cells, a gift from B. Mukhopadhyay (University of Illinois at Urbana-Champaign), were lysed by sonication and soluble cell extract was desalted on a Sephadex G-25 (Pharmacia) column. To 100 μL cell extract (7.6 mg·mL^{-1} protein) in buffer (50 mM pyridine/HCl pH 5.5, 1 mM $MgCl_2$) was added 1 μmol *rac*-phosphosulfolactate. Reactions were incubated at 70 °C for 2 h and then macromolecules were removed by precipitation with one vol. ethanol. Solute was evaporated with a stream of nitrogen gas and the dried residue was resuspended in water and purified using a Dowex 50-8X H^+ column. Sulfolactate product was derivatized and analyzed by GC-MS as described for *Methanosarcina thermophila*.

Cloning and recombinant expression of the MJ1140 gene in *E. coli*

The MJ1140 gene (encoding protein gb|AAB99140.1) was amplified by PCR from *M. jannaschii* genomic DNA using oligodeoxynucleotide primers synthesized by Gibco BRL. The MJ1140-Fwd (5′-GGTCATATGATAACTCTATGTAAC-3′) primer introduced a *Nde* I restriction site at the 5′ end of the amplified DNA whereas MJ1140-Rev (5′ 8GATCGGATCCT TATTTAAATCTAACAACTTTGC-3′) introduced a *Bam*HI site at the 3′-end. Amplified DNA specified an ATG initiator codon 99 nucleotides downstream from the hypothetical TTG initiator codon originally reported for MJ1140 [12]. PCR amplifications were performed as described previously using a 55 °C annealing temperature [8]. PCR product DNA was purified using a QIAquick spin column (QIAGEN) and then digested with *Nde* I and *Bam*HI restriction enzymes (Gibco BRL). DNA fragments were ligated into compatible sites in plasmid pET19b (Novagen) using bacteriophage T4 DNA ligase (Gibco BRL). Recombinant plasmids were transformed into *E. coli* NovaBlue (Novagen) and *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene). For expression of untagged MJ1140 protein, the *Nde* I–*Bam*HI DNA fragment containing the MJ1140 gene was subcloned into plasmid pT7-7 [13] and the recombinant plasmid was transformed into *E. coli* BL21-CodonPlus(DE3)-RIL.

Transformed *E. coli* cells were grown in 200 mL Luria–Bertani medium (Difco) supplemented with 100 mg·L^{-1} ampicillin. Cultures were shaken at 37 °C until they reached an absorbance at 600 nm of 1.0. Recombinant protein production was then induced with 28 mM lactose. After an additional 4-h incubation with shaking at 37 °C the cells were harvested by centrifugation (4000 ***g***, 5 min) and frozen at −20 °C. Induction of the desired protein was confirmed by SDS/PAGE analysis of total cellular protein.

Preparation of cell extracts and protein purification

Recombinant protein was purified by heat treatment of sonicated, soluble cell extract. Cells (2.2 g wet weight) were suspended in buffer A (11 mL) containing 5 mM imidazole, 500 mM NaCl and 20 mM Tris/HCl pH 7.9. Soluble cell extract was obtained by centrifuging sonicated cell extract (15 000 ***g***, 10 min). Native *E. coli* proteins were denatured by heating soluble extract at 70 °C for 20 min Insoluble material was removed by centrifugation at 4 °C (16 000 ***g***, 10 min).

Recombinant protein expressed with an amino-terminal polyhistidine tag ($ComB^{His}$) was purified by affinity column chromatography (1.2 × 1 cm) using Ni^{2+}-nitrilotriacetic acid conjugated agarose (QIAGEN) with isocratic elution by imidazole at room temperature. Heat-denatured soluble cell extract was applied to a column pre-equilibrated with binding buffer. The column was washed with buffer B (5 mL) containing 60 mM imidazole, 500 mM NaCl and 20 mM Tris/HCl pH 7.9. Protein was eluted in buffer C containing 380 mM imidazole, 500 mM NaCl and 20 mM Tris/HCl pH 7.9. Purified protein was analyzed by SDS/PAGE (12% polyacrylamide) with Coomassie blue or silver diamine staining. Coomassie blue-stained $ComB^{His}$ protein in SDS/PAGE gels was quantified using BSA as a standard. This purification yielded 88 μg protein at a concentration of 66 ng·μL^{-1}. Recombinant enterokinase (Novagen) was used according to the manufacturer's directions: 0.8 μg purified ComB was incubated in the supplied reaction buffer with 0.5 U enterokinase at room temperature or at 37 °C for 5–7 h (25 μL total volume). Cleavage was assayed by SDS/PAGE (12 or 15% polyacrylamide). Under these reaction conditions, the enzyme efficiently digested the Cleavage Control Protein (Novagen).

Recombinant protein expressed without an affinity tag was purified by several chromatographic separations. Cells (0.5 g wet weight) were suspended in buffer D containing 20 mM bis-Tris/HCl pH 6.5. The cell suspension was sonicated and heat-treated as described above. Heat-treated soluble cell extract was filtered through a 0.22-μm polysulfone membrane (Gelman) and then applied to a Sephadex G-25 (Pharmacia) column (10 mm × 6.5 cm) equilibrated in buffer D. Chromatography was performed in buffer D at a flow rate of 0.4 mL·min^{-1} while fractions of 0.75 mL were collected and subsequently assayed for phosphoglycolate phosphatase (PGP) activity. Standard assays (100 μL) contained 1.5 mM phosphoglycolate, 50 mM Mes-NaOH (pH 5.6), 5 mM $MgCl_2$ and 3 μL sample. Reactions were incubated for 15 min at 70 °C then inorganic phosphate was

quantified as described below. Fractions 4–7 were pooled and applied to a DEAE Sepharose FF (Pharmacia) column (20 mm × 20 cm) equilibrated in buffer D. The column was washed with 20 mL buffer D (1.3 mL·min^{-1}) before protein was eluted with a 80-mL linear gradient from buffer D to buffer E (20 mM bis-Tris/HCl pH 6.5, 1 M NaCl) at a flow rate of 1.3 mL·min^{-1}. Fractions of 1.25 mL were assayed for phosphatase activity. Six fractions (≈ 30% buffer E) containing significant amounts of phosphatase activity were pooled and concentrated by ultrafiltration (3000 × *g*, 4 °C, 1.5 h) in Centricon YM-10 devices (Millipore). Protein was desalted on a Sephadex G-25 column equilibrated in buffer F containing 20 mM Tris/HCl pH 7.5. Pooled fractions were applied to a UNO Q-1 column (Bio-Rad; 7 mm × 35 mm) equilibrated in buffer F. The column was washed with 8 mL buffer F (0.5 mL·min^{-1}) before protein was eluted with a 25-mL linear gradient (1 mL·min^{-1}) from buffer F to buffer G (20 mM Tris/HCl pH 7.5, 1 M NaCl). Fractions of 1 mL were collected and assayed for phosphatase activity. Fractions containing activity (≈ 35% buffer G) were applied to a Superose 12 H (Pharmacia) column (10 mm × 31 cm) equilibrated in 50 mM Tes-NaOH pH 7.0, 150 mM NaCl (buffer H). Fractions of 0.5 mL were collected and phosphatase activity was identified in three fractions. These fractions were pooled and then ComB protein was concentrated by ultrafiltration in a Microcon YM-10 device.

Size exclusion chromatography was performed using a Sephacryl S-300 (Pharmacia) column (10 mm × 30 cm). The column was equilibrated and operated at a flow rate of 0.4 mL·min^{-1} in buffer I containing 50 mM Tes-NaOH pH 7.0, 150 mM NaCl, 5 mM $MgCl_2$. Protein standards used to calibrate the sizing column were horse spleen apoferritin, yeast alcohol dehydrogenase, BSA, bovine erythrocyte carbonic anhydrase and horse heart cytochrome c. Eluted protein was detected by its absorbance at 280 nm and PGP activity. Molecular masses and elution volumes of standards were successfully fit to an exponential equation using nonlinear least-squares regression (SIGMA PLOT 2000, SPSS Science).

Measurement of inorganic phosphate

Inorganic orthophosphate was measured by a colorimetric assay of molybdenum blue complexes [14]. Phosphate-containing samples were centrifuged (16 000 ***g***, 1 min) then the soluble solution was diluted to 300 μL with water. Colorimetric reagent (700 μL) containing 29 μM ammonium molybdate (J. T. Baker), 0.4 M sulfuric acid and 81 mM ascorbic acid (Fisher) was added to the sample. Reactions were incubated at 50 °C for 20 min. Absorbances of the reaction products were measured at 800 nm and phosphate content was calculated based on a standard curve made using KH_2PO_4 (Fisher). Phosphate released during kinetic analyses of $ComB^{His}$ with phosphomalate was quantified using a more sensitive malachite green molybdate assay [15]. Three vols of malachite green oxalate solution (0.045% w/v) were mixed with 1 vol. ammonium molybdate solution (4.2% w/v in 4 N HCl) for 30 min. The mixture was filtered through acid-washed Whatman no. 1 paper and then Tergitol NP-10 was added to a concentration of 0.04% (v/v). Samples were diluted to 250 μL and H_2SO_4 was added to a final concentration of 1.5 M. Samples were mixed with ammonium molybdate/malachite green solution and incubated at room temperature for 10 min; phosphate product absorbances were measured at 640 nm. Samples containing acid-labile nucleotides were incubated with molybdate reagent for 5 min at 50 °C or analyzed using the malachite green–acid molybdate assay [16].

Measurement of enzyme activities

Substrates for phosphatase assays were dissolved in water and their concentrations were determined by measuring inorganic phosphate produced by incubation with 8.6 U·mL^{-1} bacterial alkaline phosphatase in 50 mM Hepes/NaOH pH 7.5 for 6 h at 37 °C. Measurements of phosphate from alkaline phosphatase-hydrolyzed samples closely agreed with values predicted from masses of pure phosphomonoester substrates, when weighing was possible. Hydrolysis by alkaline phosphatase released more inorganic phosphate from substrates than did acid hydrolysis (1 M HCl, 80 °C, 24 h) [17]: 20% more phosphate was released from phosphosulfolactate and 50% more phosphate was released from phosphoglycolate. Phosphomonoester substrates were resistant to uncatalyzed hydrolysis under both enzymatic and inorganic phosphate assay conditions. In addition to those compounds whose synthesis is described here, several commercially available compounds were tested as substrates for ComB: *O*-phospho-L-serine, *p*-nitrophenyl phosphate, *O*-phospho-L-tyrosine, *O*-phospho-DL-threonine, D(−)-3-phosphoglyceric acid, D(+)-2-phosphoglyceric acid, 2,3-diphospho-D-glycerate, D-glucose-6-phosphate, D-ribose-5-phosphate, dihydroxyacetone phosphate, phosphoglycolate, monoethyl phosphate, DL-α-glycerophosphate and β-glycerophosphate. The sodium salt of monomethyl phosphate was prepared from a solution of the di(cyclohexylammonium) salt by cation exchange chromatography using Dowex 50-8X Na^+ resin.

Phosphatase activity was measured by quantifying inorganic phosphate produced during a fixed time-point assay. Components of a 200-μL reaction included substrate, 16.5 ng ComB enzyme, 50 mM Mes-NaOH (pH 5.5) and 5 mM $MgCl_2$. Dithiothreitol (1 mM) was included in some reactions, but did not affect activity during short incubations. Reactions were preincubated for 10 min at 70 °C before the addition of substrate. Enzyme activity was stopped by the addition of 100 μL H_2SO_4 (0.5 M) and inorganic phosphate was measured in the reaction product.

Phosphosulfolactate phosphatase activity was optimized by varying reaction conditions including temperature, pH and inorganic cation concentration. Constant ionic strength buffers consisting of 0.5 M acetic acid (Fisher), 0.5 M Mes and 1 M Tris were adjusted to appropriate pH at room temperature using NaOH or HCl [18]. Buffers were diluted 10-fold in assays containing 3 mM *rac*-phosphosulfolactate and 5 mM $MgCl_2$ at 70 °C. Effects of KCl (Fisher) on phosphatase activity were tested in 50 mM Mes-NaOH (pH 5.5) with 4 mM *rac*-phosphosulfolactate and 5 mM $MgCl_2$ at 70 °C. Divalent cation requirements were tested in similar reaction conditions, using 5 mM concentrations of $MgCl_2·6H_2O$ (Fisher), $MnCl_2·4H_2O$ (Fisher), $ZnSO_4·4H_2O$ (Fisher), $NiCl_2·6H_2O$, $CoCl_2·6H_2O$ (J. T. Baker), $Fe(NH_4)_2(SO_4)_2·6H_2O$, $CaCl_2·6H_2O$ (Fisher), $CuCl_2$ (Fisher), $BaCl_2·2H_2O$ (Fisher), $CdCO_3$ (Thiokol), CuCl (Thiokol), $FeCl_3·6H_2O$ (J. T. Baker) or EDTA. The reaction

temperature optimum was determined by adding *rac*-phosphosulfolactate substrate (3 mM) to reaction mixtures pre-equilibrated in water baths. Reactions at temperatures above 70 °C were covered with a drop of paraffin oil. All reactions were carried out in enzymatic activity-limited conditions.

Initial velocities of ComB phosphatase activity were measured at substrate concentrations in the range 10 μM to 0.8 mM using 5 min fixed time-point assays. Initial velocities could not be measured accurately at lower substrate concentrations or earlier time points due to the detection limits of the inorganic phosphate assays. Initial velocity data from these substrate concentrations were successfully fit to the hyperbolic Michaelis–Menten equation and kinetic parameters were calculated using the Levenberg–Marquardt method of nonlinear least-squares regression (SIGMA PLOT 2000, SPSS Science).

Enzymatic synthesis of phosphomonoester substrates

Rabbit muscle pyruvate kinase was used to synthesize several 2-hydroxycarboxylic acid phosphate esters [19]. Reactions (200 μL) contained 10 mM 2-hydroxycarboxylic acid substrate, 2 mM ATP, 2 mM $MgCl_2$, 50 mM Tris/HCl pH 8.0, 80 mM KCl and 20 U rabbit muscle pyruvate kinase. Reactions were incubated at room temperature for 2 h. Product synthesis was inferred from assays of ADP formation (100 μL total volume) containing 0.15 mM β-NADH, rabbit muscle pyruvate kinase (0.9 U), rabbit muscle lactate dehydrogenase (1.3 U), 50 mM Hepes-NaOH pH 7.5, 0.25 mM phosphoenolpyruvate, 2 mM $MgCl_2$ and 50 μL synthesis reaction. Reactions were incubated at 37 °C for 30 min before β-NADH concentrations were measured spectrophotometrically by their absorbance at 365 nm. 2-Hydroxycarboxylic acid substrates included glycolate (*S*)-lactate, (*R*)-lactate, *rac*-glycerate, (*R*)-glycerate, (*S*)-α-hydroxyglutarate and (*S*)-malate.

Inhibition of ComB phosphatase activity

Potential inhibitors of ComB activity were tested in reactions (100 μL) containing 0.2 mM *rac*-phosphosulfolactate, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM Mes-NaOH (pH 5.5) and 0.1 μg ComB. Reactions were incubated at 70 °C for 20 min. Inorganic phosphate product was quantified as described above. Compounds tested as inhibitors included 1 mM sodium fluoride (J. T. Baker), 1 mM sodium molybdate (J. T. Baker), tartrate and 2.5 or 5 mM calcium chloride (Fisher). A 0.1-M solution of 2-carboxyethylphosphonic acid was prepared by dissolution in water and adjusting the pH to 7.0 with NaOH.

Sodium orthovanadate was dissolved in a sodium hydroxide solution (pH 10) and heated to boiling. The solution's absorbance at 260 nm was used to calculate its concentration [20]. Orthovanadate inhibition reactions (200 μL) included 33 ng ComB enzyme and 50 mM Mes-NaOH pH 5.5 preincubated at 70 °C. Orthovanadate, 5 mM $MgCl_2$, and 25 μM phosphoglycolate were preincubated separately at 70 °C and added to the enzyme to commence the reaction.

In tests for product inhibition 33 ng ComB enzyme and 50 mM Mes-NaOH pH 5.5 were preincubated at 70 °C. Separately, 5 mM $MgCl_2$ and substrate [34 μM phosphoglycolate or 148 μM (*S*)-phospholactate] were preincubated at 70 °C with 0–5 mM product [glycolate or (*S*)-lactate]. Solutions were combined to commence the reaction and free phosphate was measured after 7 min incubation at 70 °C.

Enzymatic analysis of 2-hydroxyacid enantiomers

The stereochemistry of ComB-hydrolyzed products was analyzed using stereospecific NAD^+-dependent dehydrogenases. (*R*)-Sulfolactate was quantified using the previously characterized MJ1425 2-hydroxyacid dehydrogenase (ComC) [7]. Recombinant protein was purified by affinity chromatography on a HiTrap Blue (1.6 cm × 2.5 cm) column (Pharmacia). *E. coli* cells were induced for MJ1425 expression as described previously. Cells (0.4 g wet weight) were suspended in buffer J (50 mM Hepes-NaOH, pH 7.0), sonicated and heat-treated as described for ComB. Soluble protein was applied to the affinity column and eluted with a linear gradient from buffer J to buffer K (50 mM Hepes-NaOH, 3 M NaCl pH 7.0). Purified protein (2 mg) migrated as a single band by SDS/PAGE analysis, with an apparent molecular mass of 37 kDa.

For stereochemical analysis of ComB products, reactions (100 μL) contained 250 μM or 450 μM *rac*-phosphosulfolactate, 50 mM Mes-NaOH (pH 5.6), 1 mM $MgCl_2$ and 0.16 μg $ComB^{His}$. Reactions were incubated at 70 °C for 30 min before 20 μL was removed for analysis of released inorganic phosphate. To the remaining volume was added 3 μL 1 N NaOH. Dehydrogenase assay reactions (100 μL) contained 60 μL ComB product, 50 mM Hepes-NaOH pH 7.2, 1 mM β-NAD^+, 150 μM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 100 μM phenazine methosulfate and 1 μg ComC enzyme [21]. Reactions were incubated at 70 °C for 30 min then Triton X-100 was added to a final concentration of 1% (v/v). Samples were cooled and mixed thoroughly before absorbances were measured at 590 nm and (*R*)-sulfolactate concentrations were calculated based on a standard curve derived from reactions containing known amounts of (*R*)-sulfolactate (0–400 μM). ComC did not oxidize (*S*)-sulfolactate significantly at comparable concentrations. Both sulfolactate enantiomers were purified from dicyclohexylamine salts [7] by passage through a Dowex AG-$50H^+$ column and titration with NaOH. Concentrations of sulfolactate in stock solutions were determined by oxidation with ammonium monovanadate-sulfuric acid reagent at 90 °C for 15 min [22]. Blue vanadium(IV) product was quantified by its absorbance at 775 nm and compared to standard curves derived from (*S*)-malate or (*S*)-lactate solutions. Calculated concentrations were similar to those inferred from pH titrations.

The stereochemistry of malate from ComB hydrolysis of phosphomalate enantiomers was determined using porcine heart mitochondrial L-malate : NAD^+ oxidoreductase (EC 1.1.1.37). Reactions were carried out as described above for phosphosulfolactate using 6 U (*S*)-malate dehydrogenase in 100 μL reaction volumes. Samples were incubated with enzyme for 30 min at room temperature before the addition of Triton X-100 [1% (v/v)] and absorbances were measured at 563 nm. A standard curve prepared using a solution of (*S*)-malate was used to calculate (*S*)-malate concentrations in samples.

Phosphoryl transfer reaction from phosphoglycolate

ComB was assayed for phosphotransferase activity after incubation for 7 min at 70 °C. The enzyme activity-limited reaction (500 μL) included 25 mM pyridine-HCl (pH 5.5), 5 mM $MgCl_2$, 0.1 μg ComB, 2 mM (*S*)-lactate and 320 μM phosphoglycolate. Enzyme activity was stopped by the addition of 50 μL 1 M HCl. Reactions were processed and the components derivatized with TMS for GC-MS analysis as described above.

Identification of ComB homologs, sequence alignment and phylogenetic inference

The translated sequence of *M. jannaschii* gene MJ1140 (gb|AAB99140.1) was used to query the nonredundant protein database at the National Center for Biotechnology Information using the BLASTP program (v. 2.1.2) [23]. Homologs were identified in the archaea *Methanobacterium thermoautotrophicum* (gb|AAB85671.1) and *Thermoplasma acidophilum* (emb|CAC12552.1), and in the bacteria *Thermotoga maritima* (gb|AAD35879.1), *Deinococcus radiodurans* (gb|AAF10969.1), *Synechocystis* sp. (dbj|BAA17908.1) and *Bacillus subtilis* (emb|CAA70658.1). Additional homologs were identified in incomplete genomic sequences from *Ferroplasma acidarmanus*, *Prochlorococcus marinus*, *Synechococcus* sp., *Cytophaga hutchinsonii*, *Nostoc punctiforme* (http://www.jgi.doe.gov), *Streptomyces coelicolor* (http://www.sanger.ac.uk), *Bacillus stearothermophilus* (http://www.genome.ou.edu), *Clostridium acetobutylicum* (http://www.cric.com), *Porphyromonas gingivalis* (http://www.tigr.org) and *Anabaena* sp. (http://www.kazusa.or.jp/cyano/anabaena).

Amino-acid sequences were aligned automatically using the CLUSTALW program (v. 1.81) [24]. From the alignment of 17 proteins, 206 positions were deemed to be confidently aligned. These were analyzed by protein maximum likelihood methods using the PROML program [PHYLIP (phylogeny inference package), vs. 3.6a; J. Felsenstein, Department of Genetics, University of Washington, Seattle, 2000].

RESULTS

Methanosarcina thermophila and *M. jannaschii* hydrolyze phosphosulfolactate to sulfolactate

In the proposed pathway to CoM biosynthesis, PEP is sulfonated to form phosphosulfolactate, which is then dephosphorylated, oxidized and decarboxylated (Fig. 1). Reductive thiolation of the sulfoacetaldehyde intermediate forms CoM. The oxidation of (*R*)-sulfolactate and the subsequent decarboxylation reaction have been shown to be catalyzed by *M. jannaschii* enzymes ComC (MJ1425) and ComD and ComE (MJ0255.1 and MJ0256), but phosphosulfolactate phosphatase activity has not previously been described. Incubation of *rac*-phosphosulfolactate with cell extract from *Methanosarcina thermophila and M. jannaschii* produced the expected sulfolactate product, which was derivatized and identified by GC-MS. No sulfolactate was identified in reactions incubated without phosphosulfolactate.

Identification, cloning and purification of a 2-hydroxycarboxylic acid phosphate phosphatase from *M. jannaschii*

Among the set of organisms whose complete genome sequences are known, *M. jannaschii and Methanobacterium thermoautotrophicum* are the only microbes known to produce CoM. *Bacillus subtilis* also produces sulfolactate during sporulation [25]. All three genome sequences encode homologs of the (*R*)-sulfolactate dehydrogenase (ComC), therefore we hypothesized that each would contain a homologous phosphosulfolactate phosphatase (ComB). Many genes are genetically linked to other genes in the same biosynthetic pathway [26], therefore we also hypothesized that the *comB* gene would be adjacent to a recognized CoM (or sulfolactate) biosynthetic gene. A gene adjacent to the *comDE* genes that encode sulfopyruvate decarboxylase in *M. jannaschii* (MJ0255) has homologs in all three genomes; however, this enzyme does not have demonstrable phosphatase activity (data not shown). The *yitD* homolog of MJ0255 in *Bacillus subtilis* is adjacent to another ORF (*yitC*) which also has homologs in the genomes of both methanogens. The *yitC* homologous gene from *M. jannaschii* (MJ1140) was cloned into a plasmid and expressed recombinantly in *E. coli* from a T7 RNA polymerase promoter. Heat-treated soluble cell extract from the recombinant *E. coli* had thermophilic phosphosulfolactate phosphatase activity. Similarly prepared extracts from *E. coli* cells without the recombinant MJ1140 gene did not have this activity.

Polyhistidine-tagged ComB ($ComB^{His}$) was purified by affinity chromatography on Ni-nitrilotriacetic acid resin. Purified protein analyzed by SDS/PAGE showed two bands: a primary product (32 kDa, 93% total protein) that corresponded to the expected size of $ComB^{His}$ and a secondary product (30 kDa, 7% total protein) which may be a proteolytically cleaved derivative of the primary product (Fig. 3). The two proteins coelute from strong anion exchange and size exclusion chromatography columns. Although the polyhistidine fusion tag contains an enterokinase cleavage site, both proteins resist cleavage by enterokinase at room temperature or at 37 °C in the presence of 2 M urea or 1% Triton X-100. Size exclusion chromatography of the purified protein showed a single peak with an apparent value for the native molecular mass of 30 kDa; therefore recombinant $ComB^{His}$ protein appears to be a monomer. Co-injection of $ComB^{His}$ with purified ComC protein had no effect on the elution profile of either enzyme from the size exclusion column. Although $ComB^{His}$ is expressed at high levels in *E. coli*, it is sparingly soluble ($< 100\ ng \cdot \mu L^{-1}$) and attempts to concentrate the protein solution were unsuccessful. Purified protein was stored in elution buffer at 4 °C and retained 93% activity after 3 weeks. Enzyme stored for 3 weeks at −30 °C in the presence of 50% (v/v) glycerol retained 75% of its original activity.

Recombinant ComB without the polyhistidine tag was chromatographically purified to confirm activation by divalent cations. The *comB* gene was subcloned into expression vector pT7-7 to produce a recombinant protein with the predicted native start site. ComB was purified from heat-treated recombinant *E. coli* cell extract by weak anion exchange chromatography, strong anion exchange chromatography and then size exclusion chromatography. From

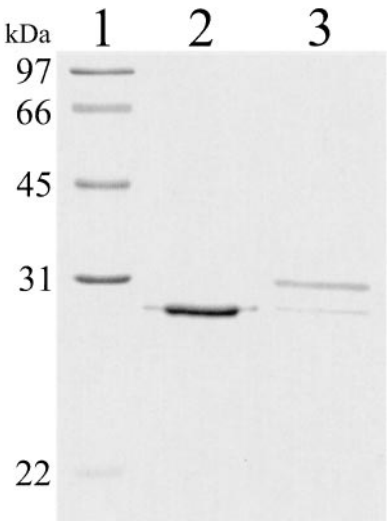

Fig. 3. Silver-stained SDS/PAGE analysis of purified ComB and ComB^{His}. The gel used was 12% monomeric acrylamide, 2.7% crosslinking monomer with the Laemmli buffer system. Lane 1, molecular mass standards; lane 2, ComB protein; lane 3, ComB^{His} protein (top band) and putative degradation product purified by nickel affinity chromatography.

lactose-induced *E. coli* cells (0.5 g wet weight) was purified 7 μg ComB in 150 μL final volume. Purified protein migrated as a single band on an SDS/PAGE gel with an apparent molecular mass of 30 kDa (Fig. 3).

2-Hydroxycarboxylic acid phosphate phosphatase activity of ComB

Affinity purified ComB^{His} efficiently dephosphorylates up to 100% of available *rac*-phosphosulfolactate at 70 °C (Table 1). The enzyme is most active at pH 5.5, 75 °C and requires Mg^{2+} at concentrations approximately equimolar with substrate (Fig. 4A and B). Ni^{2+}, Co^{2+}, Mn^{2+} and Zn^{2+} can fully substitute for the Mg^{2+} requirement. ComB^{His} is less active when Cu^{2+} is substituted for Mg^{2+}. Cd^{2+}, Fe^{3+}, Cu^{+}, Fe^{2+} and Ca^{2+} cannot substitute for Mg^{2+}. Except for Ba^{2+}, all of these 'inactive' metals inhibit ComB^{His} activity in the presence of equimolar Mg^{2+} (Fig. 4C). As expected, EDTA inhibits ComB^{His} activity. Enzyme activity is not stimulated by monovalent salts (KCl) or by the addition of dithiothreitol. This enzyme's specific activity at 70 °C (pH 5.5) is 48 μmol *rac*-phosphosulfolactate hydrolyzed·min^{-1}·mg^{-1} ComB^{His}.

ComB without an affinity tag was activated by a similar set of divalent cations. In reactions (70 °C) containing 0.1 μg ComB, 2 mM *rac*-phosphosulfolactate, 5 mM divalent cation metal and 50 mM Mes-NaOH pH 5.6, the specific activity of ComB in the presence of 5 mM $MgSO_4$ was 8 μmol·min^{-1}·mg^{-1} compared to 7 μmol·min^{-1}·mg^{-1} for ComB^{His}. Differences between ComB and ComB^{His} specific activities were not significant. Specific activities relative to Mg^{2+} activites for ComB and ComB^{His}, respectively, were 360% and 570% for Ni^{2+} 290% and 220% for Co^{2+}, 90% and 170% for Zn^{2+} and 80% and 50% for Mn^{2+}.

In addition to hydrolyzing phosphosulfolactate, ComB^{His} efficiently dephosphorylates (*S*)-phospholactate and phosphoglycolate (Table 1). Pyruvate kinase was used to enzymatically synthesize *rac*-2-phosphoglycerate from *rac*-glycerate. This product was hydrolyzed by ComB^{His} whereas the phosphorylated product of (*R*)-glycerate was not hydrolyzed. Therefore (*S*)-2-phosphoglycerate is a substrate for ComB hydrolysis.

The enzyme does not efficiently hydrolyze (*R*)-2-phospholactate or (*R*)-2-phosphoglycerate or hydroxyphosphate polyols such as DL-α-glycerophosphate or β-glycerophosphate. Neither does it dephosphorylate *p*-nitrophenyl phosphate, monomethyl phosphate, monoethyl phosphate, D-glucose-6-phosphate or *O*-phospho-amino acids such as *O*-phospho-L-serine, *O*-phospho-L-tyrosine or *O*-phospho-DL-threonine. Therefore the *M. jannaschii* ComB is a metal-dependent acid phosphatase of 2-hydroxycarboxylic acid phosphates.

Kinetic analysis of ComB activity

In the presence of 5 mM $MgCl_2$, initial velocities of ComB^{His} hydrolysis of *rac*-phosphosulfolactate (10–800 μM) fit the Michaelis–Menten–Henri kinetic model for single substrate reactions (Fig. 5) [27]. The ComB^{His} enzyme dephosphorylates phosphoglycolate, (*S*)-phospholactate and *rac*-phosphosulfolactate equally efficiently (Table 1). (*S*)-Phosphomalate and (*R*)-phosphomalate are both hydrolyzed with lower K_m^{app} values but turnover numbers are comparable to those of the other substrates.

Inhibition of ComB activity

ComB phosphatase activity requires 2-hydroxycarboxylic acid phosphates and divalent cations, which may form an activated substrate complex. Compounds that mimic or disrupt this complex inhibit ComB activity. These include

Table 1. Kinetics of the activity of affinity purified ComB^{His}. Values are means ± standard deviations ($n = 5$). ND, not determined.

Substrate	K_m^{app} (μM)[a]	k_{cat}/K_m ($M^{-1}·s^{-1}$)	Substrate conversion (%)[b]
rac-Phosphosulfolactate	63 ± 10	4.1×10^5	99 ± 2.2%
(*S*)-Phospholactate	148 ± 44	6.2×10^5	103 ± 3.4%
(*R*)-Phospholactate	ND	ND	7 ± 2.8%
(*S*)-Phosphomalate	9 ± 5	1.4×10^6	96 ± 8.6%
(*R*)-Phosphomalate	11 ± 5	1.0×10^6	96 ± 5.3%
Phosphoglycolate	34 ± 11	9.2×10^5	100 ± 5.3%

[a] Apparent K_m parameters and their standard errors were derived from nonlinear regression analyses. [b]The maximum quantity of substrate hydrolyzed under conditions of excess ComB enzymatic activity.

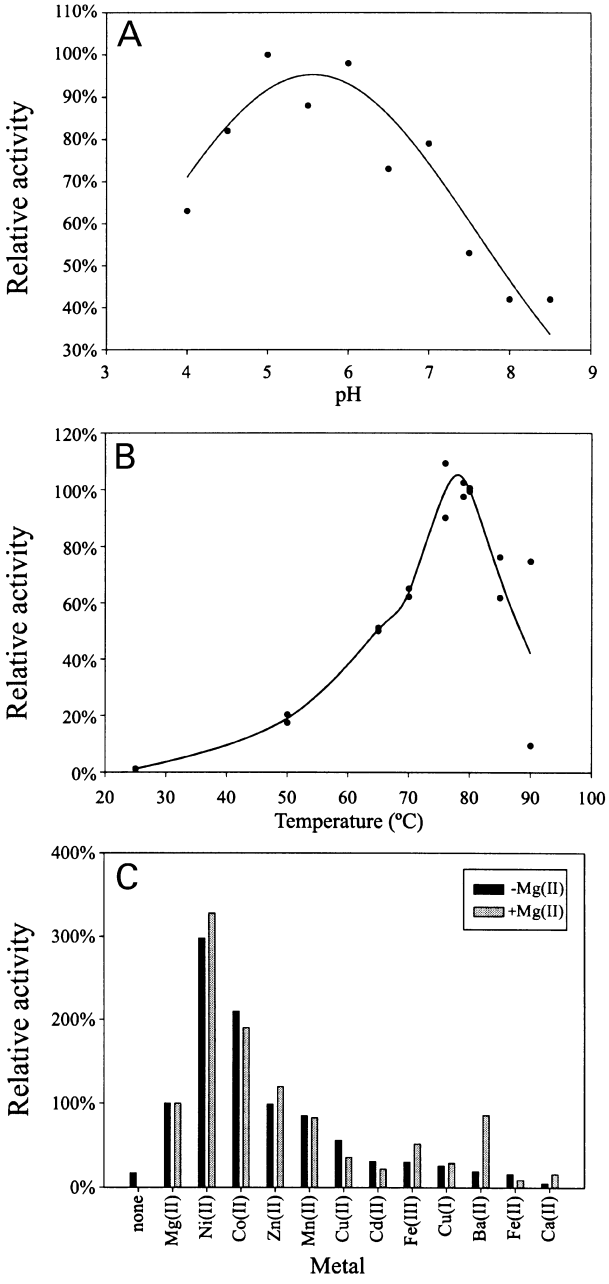

Fig. 4. Activity of ComB^{His} hydrolyzing *rac*-phosphosulfolactate. (A) Activity in constant ionic strength pH buffers relative to a maximum specific activity of 49 μmol·min^{-1}·mg^{-1}. Reactions containing 5 mM $MgCl_2$ and 3 mM *rac*-phosphosulfolactate were incubated at 70 °C. (B) Enzyme activity as a function of temperature. A maximum specific activity of 35 μmol min^{-1} mg^{-1} was recorded at 79 °C. Reactions contained 5 mM $MgCl_2$, 300 μM *rac*-phosphosulfolactate and 50 mM Mes-NaOH pH 5.5. (C) Effects of 5 mM metal ions on activity relative to specific activity in the presence of 5 mM Mg^{2+} (17 μmol·min^{-1}·mg^{-1}). Stimulation of activity relative to Mg^{2+}. Activity in the presence of 5 mM $MgCl_2$, relative to activity with magnesium ions alone (17 μmol min^{-1} mg^{-1}). Reactions including 2 mM *rac*-phosphosulfolactate, 50 mM Mes-NaOH (pH 5.5) and 5 mM metal cation were incubated at 70 °C.

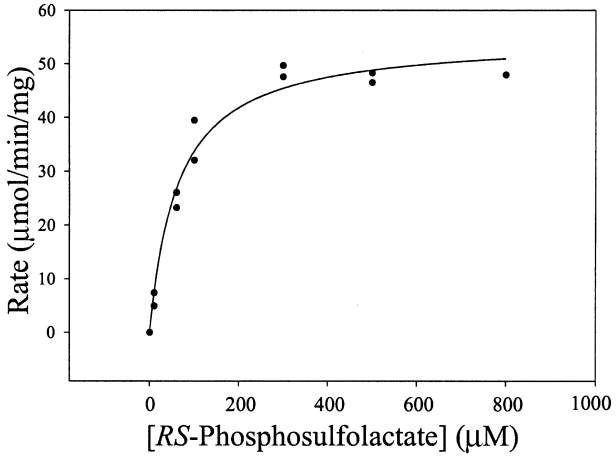

Fig. 5. Kinetic analysis of ComB *rac*-phosphosulfolactate phosphatase activity. Initial rates are shown as micromoles of phosphate produced per min per mg ComB^{His} enzyme. Reactions including 5 mM $MgCl_2$ and 50 mM Mes-NaOH pH 5.5 were incubated at 70 °C.

chelators of divalent ions (EDTA) and metal ions such as Ca^{2+} ions that adopt different coordination geometries. Millimolar concentrations of sodium fluoride or sodium molybdate, common phosphatase inhibitors, do not affect ComB activity. ComB is inhibited by vanadate, a phosphate analog [28]. In a reaction with 30 μM phosphoglycolate, vanadate inhibits ComB^{His} activity with a 50% inhibitory concentration (IC_{50}) of 0.44 ± 0.15 mM, comparable to other acid phosphatases [29]. 2-Carboxyethylphosphonate, a phosphoglycolate analog, is a weak inhibitor of ComB^{His} PGP activity, with an IC_{50} of 0.72 ± 0.23 mM in reactions containing 25 μM phosphoglycolate. *O*-Phospho-L-serine (5 mM) does not inhibit PGP activity. Carboxylic acid reaction products [(*S*)-lactate or glycolate] do not significantly inhibit (*S*)-phospholactate or PGP activity at low millimolar concentrations.

Probing a possible enzymatic mechanism

Studies of the metal-catalyzed nonenzymatic hydrolysis of 2-hydroxycarboxylic acid phosphate esters suggested the formation of a pentacovalent cyclic phosphate intermediate that resolves into a phosphoanhydride [30]. To ascertain whether the ComB phosphatase mechanism involves substrate cyclization, i.e. the transfer of phosphate from the 2-hydroxyl to the carboxyl moiety, five oxygens of (*S*)-phospholactate were exchanged for ^{18}O by heating in acidic $H_2^{18}O$. After cleavage of the sample with the ComB^{His} phosphatase the recovered phosphate had an oxygen distribution of 26.8 $^{18}O_0$, 25.8 $^{18}O_1$, 25.4 $^{18}O_2$, and 22.0 $^{18}O_3$ and the recovered substrate had the same distribution as that found in the starting material. Phosphate exchanged under the conditions described to produce the labeled substrate exchanges all four oxygens. Based on the calculated ^{18}O content of the substrate's carboxyl group the incorporation of the label from the carboxyl group into the phosphate group had to occur with $< 2\%$ isotopic retention. Therefore the dephosphorylation reaction does not proceed by the hydrolysis of the C_1-O bond in the proposed phosphoanhydride intermediate.

Phosphoryl transfer activity

Phosphatases that form covalent intermediates during catalysis can hydrolyze the phosphoenzyme bond (releasing inorganic phosphate) or transfer the phosphate back to an organic substrate moiety (regenerating substrate). Infrequently, the phosphoenzyme may exchange the dephosphorylated substrate for an exogenous nucleophile and then phosphorylate it, resulting in a net transfer of phosphate from one organic molecule to another [31]. In the presence of high concentrations of (*S*)-lactate $ComB^{His}$ can dephosphorylate phosphoglycolate and produce 2-phospholactate. GC-MS analysis of reaction products detected phospholactate at 4.5% of the remaining phosphoglycolate concentration. No phospholactate was produced in reactions missing $ComB^{His}$ or in reactions with sufficient $ComB^{His}$ to hydrolyze both phosphoglycolate and phospholactate product.

Stereospecificity of ComB

In incubations with excess $ComB^{His}$ enzyme, *rac*-phosphosulfolactate is completely hydrolyzed (Table 1). Because the organic synthesis of phosphosulfolactate involves the uncatalyzed condensation of bisulfite with prochiral phosphoenolpyruvate, the phosphosulfolactate product should be a racemic mixture. Furthermore, the C-2 proton of *rac*-phosphosulfolactate does not exchange in D_2O (pH 5.5) during prolonged incubations 80 °C (data not shown). A stereospecific NAD^+-dependent 2-hydroxyacid dehydrogenase (ComC) was used to quantify (*R*)-sulfolactate produced by ComB hydrolysis of *rac*-phosphosulfolactate. *rac*-Phosphosulfolactate was hydrolyzed completely [96 ± 7% (± SD); $n = 9$)] and one-half of the sulfolactate product (48 ± 14%) was oxidized by ComC. Therefore the phosphosulfolactate was a racemic mixture and the ComB phosphatase hydrolyzes both enantiomers. We have not found a useful synthetic scheme to make stereochemically pure (*R*)-phosphosulfolactate and the enantiomers are not readily separated. However, both enantiomers of 2-phosphomalate were synthesized to produce compounds with two anionic functional groups comparable to phosphosulfolactate (Fig. 2). ComB hydrolyzes both (*S*)-phosphomalate and (*R*)-phosphomalate at comparable rates (Table 1). Malate released by ComB was assayed using stereospecific NAD^+-dependent (*S*)-malate dehydrogenase. The ComB-hydrolyzed product of (*S*)-phosphomalate was oxidized by this enzyme (122 ± 26% NAD^+ reduced per inorganic phosphate). In contrast the product of (*R*)-phosphomalate hydrolysis was not recognized by the dehydrogenase (3 ± 3%). These results confirm the stereochemistry of the synthesized phosphomalate enantiomers.

Sequence analysis of ComB homologs and phylogenetic inference

An alignment of the *M. jannaschii* ComB amino-acid sequence with homologous sequences shows that most amino acid positions are not conserved, especially in the carboxy-terminal third of the protein (Fig. 6). The few conserved residues in *M. jannaschii* ComB with possible catalytic or binding importance are Arg24, four acidic residues (Asp21, Asp145, Asp146, Asp197) three hydroxyl side chain amino acids (Thr95, Thr96, Thr99) and two asparagine residues (Asn77, Asn97). This enzyme has no conserved cysteine or histidine residues, which form covalent phosphoryl-enzyme intermediates in some phosphatases [32].

The considerable sequence divergence among members of this phosphatase family complicates phylogenetic inference. The best phylogenetic tree inferred by maximum likelihood analysis (Fig. 7) shows little congruence with standard organismal phylogenies [33]. Except for cyanobacteria, few major taxonomic lineages have vertically inherited the gene.

DISCUSSION

Although phosphatases have traditionally been classified by their substrate specificities, recent structural studies suggest that they are most usefully grouped by catalytic mechanism and active site geometry, in the larger context of hydrolase and transferase enzymes [34,35]. The ComB phosphatase described here has no significant sequence similarity to previously characterized enzymes, therefore understanding its mechanism and tertiary structure will be essential to deducing the gene's evolutionary history. The nonenzymatic hydrolysis of monoanion phosphate monoesters proceeds most rapidly at pH 4, via a dissociative transition state [36]. A low pH promotes the protonation of the hydroxyl leaving group and cleavage of the P–O bond is concurrent with phosphate transfer to a nucleophile (typically water) [37].

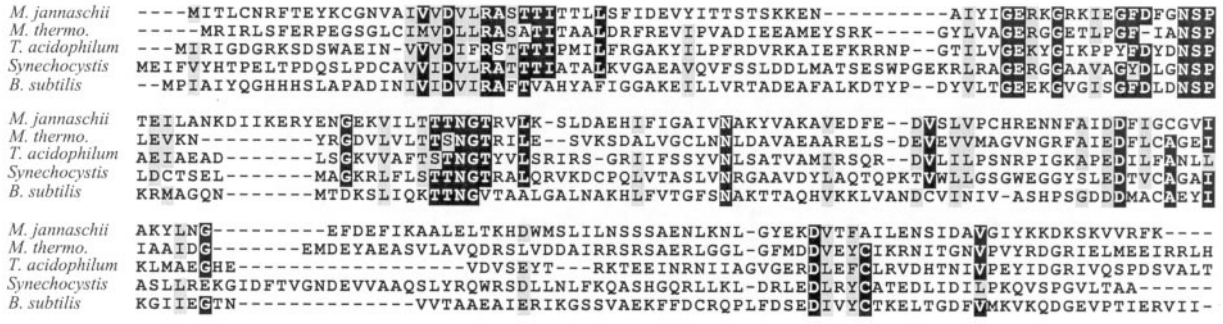

Fig. 6. Alignment of *M. jannaschii* ComB sequence with homologs from *Methanobacterium thermoautotrophicum*, *Thermoplasma acidophilum*, *Synechocystis* sp. and *Bacillus subtilis*. Positions of conserved residues are in inverted text whereas positions of similar residues are shaded gray.

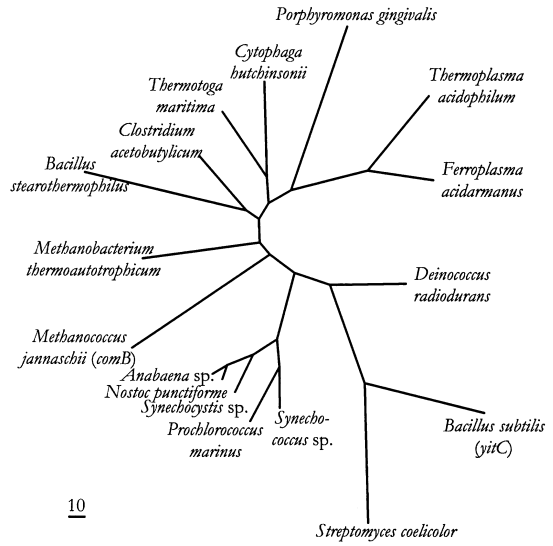

Fig. 7. Phylogeny of ComB homologs. The scale bar represents 10 amino acid replacements per 100 positions.

Acid phosphatase enzymes catalyze this reaction using a general acid residue that protonates the leaving group, basic residues or metal cations that enhance the phosphate group's electrophilicity and a nucleophilic residue that binds phosphate to form a phosphoenzyme complex [32,34,38]. ComB hydrolysis probably proceeds through similar intermediates: results from the experiments described here using ComB are consistent with this acid phosphatase mechanism.

The most comparable enzyme to ComB is PGP, which has been found in autotrophic bacteria, plant chloroplasts and mammalian tissue. In plants and bacteria PGP hydrolyzes one product of the ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) whereas in mammals PGP may hydrolyze a side product of pyruvate kinase [16]. Representative plant and mammalian enzymes have biochemical properties similar to ComB: all are metal-dependent phosphatases, with comparable kinetic parameters for phosphoglycolate (Table 2).

Although the biochemically characterized plant and mammalian PGPs have not yet been cloned and sequenced, plant cDNA libraries include sequences similar to the PGP gene from the bacterium *Alcaligenes eutrophus* [39]. The sequenced PGPs are members of a well-characterized group of hydrolases, the haloacid dehalogenase (HAD) superfamily [40]. Similar to other enzymes in the HAD superfamily, the plant PGP forms a phosphoenzyme intermediate [16]. HAD-type phosphatases require a metal ion (presumably Mg^{2+}) in an octahedral coordination geometry. Coordinating ligands for the magnesium ion include three aspartate side chain carboxylates, a main chain carbonyl, a water molecule and the phosphate oxygens of a substrate [41]. Nucleophilic attack by one of the aspartate carboxylate oxygens forms an acyl-phosphate intermediate, displacing the hydroxyl product substrate. The alkaline labile acyl-phosphate is hydrolyzed by a water molecule, releasing inorganic phosphate. The aspartate residues, along with lysine and serine/threonine residues that hydrogen bond with the phosphate, form characteristic motifs in all HAD-type phosphatases.

Although sequences of the *M. jannaschii* phosphosulfolactate phosphatase and its homologs are dissimilar to those of the HAD-type phosphatases, the group of evolutionarily conserved amino acid residues is similar (Fig. 6). Both PGP and ComB can catalyze transphosphorylation reactions, indicative of phosphoenzyme intermediates [31]. As observed for the HAD-type tobacco leaf PGP and rabbit muscle magnesium-dependent phosphatases, phosphohydrolysis by ComB is activated efficiently by several metal ions (Mg^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+}) that all have similar ionic radii and coordination geometries [29,42]. Therefore the ComB protein may catalyze hydrolysis through a similar Mg^{2+}-dependent phosphoenzyme reaction mechanism.

Most enzymes are stereospecific: they recognize only one stereoisomer of a chiral substrate and catalyze its conversion into a single stereoisomer product. When exposed to the 'wrong' stereoisomers, these enzymes either fail to bind the molecules or bind them in a nonproductive complex. Alternatively, many nonspecific enzymes, such as alkaline phosphatase or alcohol dehydrogenase, are also nonstereospecific: they are active with both substrate enantiomers [43]. Remarkably, ComB dephosphorylates both enantiomers of some substrates, but only one enantiomer of others. It efficiently hydrolyzes both enantiomers of phosphosulfolactate and phosphomalate, but hydrolyzes only (*S*)-phospholactate and not (*R*)-phospholactate. We propose that this partial stereospecificity results from the pseudo-symmetry of the phosphosulfolactate and phosphomalate enantiomers.

Table 2. Comparison of plant and mammalian PGPs with *M. jannaschii* ComB.

	Tobacco leaf PGP[a]	Spinach leaf PGP[b]	Human erythrocyte PGP[b]	*M. jannaschii* ComB
Monomeric MW	20 kDa	35 kDa	35 kDa	30 kDa
Oligomeric state	Tetramer	Dimer	Dimer	Monomer
Cofactors	Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}	Mg^{2+}, ND	Mg^{2+}, ND	Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Ni^{2}
pI	3.8	ND	ND	5.5[c]
pH optimum	5.5–7.5	ND	6–7	5.5
K_M phosphoglycolate	26 μM	30 μM	61 μM	34 μM
K_M ethyl phosphate	150 μM	640 μM	72 μM	–[d]
K_M phospholactate	–[d]	–[d]	53 μM	150 μM

[a] Data from [48,49]. [b]Data from [50]. [c]Theoretical pI calculated using http://expasy.cbr.nrc.ca/tools/pi_tool.html. [d]Enzyme does not hydrolyze this substrate. ND, Not determined.

Sterically and electronically (*R*)-phosphosulfolactate and (*S*)-phosphomalate are similar to reversed orientation (*S*)-phosphosulfolactate and (*R*)-phosphomalate molecules (Fig. 2). Therefore ComB may bind the enantiomers in inverse orientations to form productive complexes. A similar mechanism has been proposed to explain how soybean lipoxygenase-1 oxidizes linoleic acid to form both (9*S*)- and (13*S*)-hydroperoxides [44,45].

The physiological activity of the *M. jannaschii* ComB protein is proposed to be phosphosulfolactate phosphohydrolysis due to the enzyme's kinetic efficiency and the operonal association of a *comB* homolog with other CoM biosynthetic genes in *Bacillus subtilis*. Nevertheless, alternative substrates may be present in *M. jannaschii* as an intermediate in cofactor F_{420} biosynthesis [(*S*)-phospholactate] [46] or a product of archaeal RubisCO (phosphoglycolate) [47]. We are not aware of any reports identifying 2-phosphomalate in a metabolic pathway. The construction of a *comB* mutant will be a useful test of the methanogen's predicted auxotrophy for CoM.

The surprisingly broad phylogenetic distribution of phosphosulfolactate phosphatase homologs suggests that the gene has been subject to extensive horizontal transfer (Fig. 7). Homologs in each of the cyanobacterial genomes could function as PGPs, redundant to the HAD-type PGP in hydrolyzing that product of RubisCO. Other bacterial and archaeal homologs may have been recruited for novel biosynthetic pathways requiring the hydrolysis of a 2-hydroxycarboxylic acid phosphate. If genes encoding broad specificity enzymes are most likely to be transferred laterally and assimilated into cells' metabolisms then *comB* homologs could have been recruited into a myriad of new pathways.

Similar to the eclectic distribution of *comB* homologs in bacteria and archaea, the other recognized genes involved in CoM biosynthesis also have convoluted histories. The proposed (*R*)-sulfolactate dehydrogenase (ComC) and the two subunits of the sulfopyruvate decarboxylase (ComD and ComE) have close homologs in many other organisms not known to produce CoM. Few genome sequences of nonmethanogens encode multiple enzymes from the CoM biosynthetic pathway. Either many other organisms have acquired single genes from an ancient CoM biosynthetic pathway or this pathway was itself created from genes encoding broad specificity enzymes, recruited piecemeal to synthesize the smallest essential coenzyme of methanogenesis.

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