Microbial pathway for anaerobic 5′-methylthioadenosine metabolism coupled to ethylene formation

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Edited by Bob B. Buchanan, University of California, Berkeley, CA, and approved October 20, 2017 (received for review June 29, 2017)

Numerous cellular processes involving S-adenosyl-L-methionine result in the formation of the toxic by-product, 5′-methylthioadenosine (MTA). To prevent inhibitory MTA accumulation and retain biologically available sulfur, most organisms possess the “universal” methionine salvage pathway (MSP). However, the universal MSP is inherently aerobic due to a requirement of molecular oxygen for one of the key enzymes. Here, we report the presence of an exclusively anaerobic MSP that couples MTA metabolism to ethylene formation in the phototrophic bacteria Rhodospirillum rubrum and Rhodopseudomonas palustris. In vivo metabolite analysis of genetic deletion strains demonstrated that this anaerobic MSP functions via sequential action of MTA phosphorylase (MtnP), 5′-(methylthio)ribose-1-phosphate isomerase (MtnA), and an annotated class II aldolase-like protein (Ald2) to form 2-(methylthio)acetalddehyde as an intermediate. 2-(Methylthio)acetalddehyde is reduced to 2-(methylthio)ethanol, which is further metabolized as a usable organic sulfur source, generating stoichiometric amounts of ethylene in the process. Ethylene induction experiments using 2-(methylthio)ethanol versus sulfate as sulfur sources further indicate anaerobic ethylene production from 2-(methylthio)ethanol requires protein synthesis and that this process is regulated. Finally, phylogenetic analysis reveals that the genes corresponding to these enzymes, and presumably the pathway, are widespread among anaerobic and facultatively anaerobic bacteria from soil and freshwater environments. These results not only establish the existence of a functional, exclusively anaerobic MSP, but they also suggest a possible route by which ethylene is produced by microbes in anoxic environments.

**Significance**

Sulfur is an essential element required by all organisms. Therefore, salvage of wasteful, sulfur-containing cellular by-products can be critical. Methionine salvage pathways for organisms living in oxic environments are well established. However, if and by what mechanisms organisms living in anoxic environments can regenerate methionine from such by-products remain largely unknown. This work identifies a strictly anaerobic methionine salvage pathway, the key genes for which appear to be widespread among obligate and facultatively anaerobic bacteria. Strikingly, this pathway also results in the formation of ethylene gas, a key plant hormone and signaling molecule. Anoxic environments routinely accumulate biologically produced ethylene at significant levels, but the organisms and mechanisms responsible have been slow to emerge. This study provides one possible route.


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1711625114/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1711625114
Methanocaldococcus jannaschii and bacteria such as Pseudomonas aeruginosa, this process is performed by a separate MTA deaminase and 5′-methylthioinosine phosphorylase, resulting in 5-(methylthio)ribose-1-P and hypoxanthine (14, 15). However, the ability to further metabolize 5-(methylthio)ribose or 5-(methylthio)ribose-1-P in anoxic environments is largely unknown (7, 9).

Recently, the first oxygen-independent MSP, the “MTA-isoprenoid shunt,” was fully characterized, which functions both aerobically (16) and anaerobically (17) in the facultative anaerobe Rhodospirillum rubrum (Fig. 1, green arrows). As with the universal MSP, MtnP and MtnA first sequentially catalyze the conversion of MTA to 5-(methylthio)ribulose-1-P. However, then a Rubisco-like protein (RLP) from the DeepYkr clade (18, 19) (Fig. 1, enzyme M) followed by a cupin-type 1-(methylthio)xylulose-5-P methylsulfurylase (20, 21) (Fig. 1, enzyme N) functions to catalyze formation of S-(methylthio)glutathione and 1-deoxyxylulose-5-P for isoprenoid biosynthesis from 1-(methylthio)xylulose-5P (Fig. 1, compounds 11 and 12). After reduction of the glutathione-methylthio adduct, the liberated methanethiol (Fig. 1, compound 13) is conjugated to O-acetyl-L-homoserine via O-acetyl-homoserine sulfhydrylase (Fig. 1, enzyme O) to regenerate L-methionine (16). Moreover, other variations of this pathway may exist, as some bacteria contain all of the requisite genes of the MTA-isoprenoid shunt in a single gene cluster with the cupin gene replaced by a transketolase proposed to function in methanethiol release from 1-(methylthio)xylulose-5-P (16, 22). While discovery of the MTA-isoprenoid shunt clearly established the existence of an anaerobic MSP in bacteria, it was nevertheless dispensable under anaerobic growth conditions in R. rubrum. Indeed, there were strong indications of an additional, exclusively anaerobic MSP, as strains containing knockouts of the MTA-isoprenoid shunt genes were still viable for anaerobic growth with MTA as the sole sulfur source (17, 23, 24). Here, we describe an exclusively anaerobic MSP that couples MTA metabolism to ethylene production in the purple nonsulfur α-proteobacteria Rhodosporillum rubrum and Rhodopseudomonas palustris (this study). Gene names are given in italics by each catalyzed reaction. Letters correspond to enzyme name, and numbers correspond to compound name.

Results

Sulfur Salvage Stimulates Anaerobic Ethylene Production. By virtue of the fact that inactivation of the R. rubrum MTA-isoprenoid shunt genes (Fig. 2, mtnP, mtnA, rlpl, cupin) results in loss of aerobic growth on MTA as sole sulfur source (16), whereas deletion of the mtnA, rlpl, or cupin genes had little or no effect on anaerobic MTA-dependent growth (17, 23) (SI Appendix, Fig. S1 and Table S3), it became clear that R. rubrum contains
additional mechanisms to recycle MTA via anaerobic-specific routes. Only the \textit{R. rubrum} MTA phosphorylase deletion strain (\textit{ΔmtnP}) was unable to grow anaerobically on MTA as the sole sulfur source (\textit{SI Appendix}, Fig. S1). Similarly, inactivation of homologous MTA-isoprenoid shunt genes in \textit{R. palustris} (Fig. 2) had little or no effect on anaerobic \textit{R. palustris}-dependent growth either, save for the MTA phosphorylase deletion strain (\textit{ΔmtnP}), which exhibited a slow-growth phenotype (\textit{SI Appendix}, Fig. S1 and Table S3). The existence of anaerobically expressed iso-zymes of the MTA-isoprenoid shunt appears unlikely based on homology searches against the \textit{R. rubrum} and \textit{R. palustris} genomes. Furthermore, inactivation of the \textit{R. rubrum} RLP results in a loss of methanethiol production when growing anaerobically on MTA as sole sulfur source, indicating inactivation of the MTA-isoprenoid shunt beyond this point (24) (Fig. 1, enzyme M).

To determine whether volatile compounds other than methanethiol were produced by putative anaerobic MTP(s), we performed GC-MS headspace analysis of the \textit{R. rubrum} RLP deletion strain (\textit{Δrpl1}) grown anaerobically using 1 mM sulfate or MTA as the sole sulfur source. Surprisingly, ethylene gas increased nearly 1,000-fold in the headspace of cultures grown on MTA versus sulfate (\text{C}_2\text{H}_4\text{ abundance on MTA, } 3.5 \pm 2.6 \times 10^{-9} \text{ mol per L headspace}; \text{C}_2\text{H}_4\text{ abundance on sulfate, } 3.9 \pm 3.2 \times 10^{-9} \text{ mol per L headspace}; n = 2). Ethylene was validated by GC-MS via spiking the headspace gaseous environment with ethylene standards (\textit{SI Appendix}, Fig. S2).

This stimulation of anaerobic ethylene production in cells grown on MTA versus sulfate indicated a link between the anaerobic MTP(s) and ethylene synthesis. Therefore, we grew \textit{R. rubrum} and \textit{R. palustris} wild-type and \textit{Δrpl1} strains under decreasing amounts of sulfate to up-regulate sulfur-salvage pathways. Ethylene produced throughout growth was measured by GC (\textit{SI Appendix}, Figs. S3 and S4). In all strains, ethylene was maximally stimulated during growth on low amounts (100 \mu M) of sulfate. Additionally, the \textit{R. rubrum} \textit{Δrpl1} strain produced ~200-fold more ethylene and the \textit{R. palustris} \textit{Δrpl1} strain produced approximately twofold more ethylene than their respective wild-type counterparts, suggesting that ethylene formation was linked to MTA metabolism via mechanisms different from the MTA-isoprenoid shunt.

**Anaerobic Ethylene-Coupled MTA Metabolism Requires the Gene Products of \textit{mtnP}, \textit{mtnA}, and \textit{ald2}**

Known MTA metabolism genes in \textit{R. rubrum} and \textit{R. palustris} are primarily localized to two separate gene clusters (25, 26) (Fig. 2). In addition, both organisms possess multiple forms of \textit{O-acetyl-homoserine sulhydrylase} (Fig. 1, enzyme O), the final enzyme of the MTA-isoprenoid shunt, and these enzymes may also function in \text{t-cysteine} and \text{t-homocysteine} metabolism from hydrogen sul-fide (27). Interestingly, the \textit{R. rubrum} gene cluster for \textit{mtnP} and \textit{mtnA} also contained an annotated class II aldolase-like gene (\textit{ald2}), whose start codon overlapped the \textit{mtnA} stop codon by 1 nt (Fig. 24). This \textit{ald2} gene encoded a putative 221-aa, 23.9-kDa protein of unknown function with strong sequence homology to the \textit{boma fide} \textit{E. coli} \text{t-fuculose-1-P aldolase} (\textit{FucA}) (52% identity; E value, 4 × 10^{-6}). Blastp homology search against the \textit{R. palustris} genome revealed a similar \textit{ald2} gene (51% identity; E value, 9 × 10^{-6}) 201.6 kbp upstream from the \textit{mtnP} and \textit{mtnA} genes (Fig. 24). Neither \textit{R. rubrum} nor \textit{R. palustris} possess the other \textit{fuc} gene clusters, \textit{fucO} and \textit{fucP/K}, for fuculose metabolism (28). Therefore, we further explored the link between anaerobic MTA metabolism and ethylene production by individually inactivating genes \textit{mtnP}, \textit{mtnA}, \textit{ald2}, \textit{rpl1}, and \textit{cupin} in both \textit{R. rubrum} and \textit{R. palustris} and quantified the total amount of ethylene produced when cells were grown anaerobically to stationary phase with 1 mM MTA, \textit{t-methionine}, or sulfate as sole sulfur source.

In all strains grown to stationary phase on sulfate or \textit{t-methionine}, total ethylene produced was between 0.05 and 0.2 \text{µmol of ethylene per L culture per culture optical density measured at } 660 \text{ nm (µmol/L/OD}_{660\text{nm}}) (\text{Fig. 3 A} and B). When wild-type cells were grown on MTA as sole sulfur source, ethylene levels were elevated 75-fold in \textit{R. rubrum} and 120-fold in \textit{R. palustris} compared with sulfate or \textit{t-methionine} controls (Fig. 3 A and B, gray). By contrast, MTA-grown \textit{ΔmtnP}, \textit{ΔmtnA}, and \textit{Δald2} strains of both organisms produced only low levels of ethylene, similar to \textit{t-methionine} or sulfate controls, save for the \textit{R. rubrum} \textit{ΔmtnP} strain, which exhibited no growth, and hence, no ethylene production from MTA (\text{Fig. 3 A and B, blue, green, orange}). To eliminate the possibility of polar effects introduced during in-activation of each gene, we complemented \textit{R. rubrum} and \textit{R. palustris} \textit{ΔmtnP}, \textit{ΔmtnA}, and \textit{Δald2} strains with each respective gene expressed \textit{in trans} from a complementation plasmid. In each case, ethylene production was restored to or above wild-type levels, and MTA-dependent growth was restored to wild-type growth rates in the \textit{R. rubrum} and \textit{R. palustris} \textit{ΔmtnP} strains (\textit{SI Appendix}, Table S3). In addition, the \textit{ald2} gene from \textit{R. palustris} appeared to function similarly to its \textit{R. rubrum} homolog, as the \textit{R. palustris} \textit{ald2} gene expressed heterologously in a \textit{R. rubrum} \textit{Δrpl1/Δald2} background produced twofold more ethylene than the same strain complemented with the \textit{R. rubrum} gene, or the \textit{R. rubrum} \textit{Δrpl1} strain with an intact \textit{ald2} gene (Table 1).

When the \textit{rpl1} or \textit{cupin} gene of the MTA-isoprenoid shunt was inactivated, ethylene production from MTA-grown cells increased by...
~10-fold in R. rubrum and ~3-fold in R. palustris compared with the wild-type strain of each respective organism (Fig. 3A and B, red and brown), consistent with the sulfate limitation experiments (SI Appendix, Figs. S3 and S4). Coordinately, for Δrpl1 or Δcupin strains grown on MTA versus sulfate, ~300-fold more ethylene was produced by R. rubrum and ~75-fold more by R. palustris, consistent with GS-MT observations (Fig. 3A and B, red and brown). Subsequent inactivation of the ald2 gene in the R. rubrum Δrpl1 strain resulted in a 40-fold decrease in ethylene production during growth on MTA (Fig. 3A, tan), similar to the 40-fold decrease observed in the R. rubrum Δald2 strain compared with wild type, further confirming the role of Ald2 in an anaerobic ethylene-forming MSP that is independent from the MTA-isoprenoid shunt. We also inactivated the second R. palustris RLP gene (Δopl2 strain), which is homologous to the Chlorobaculum tepidum RLP from the Photo RLP clade (18). In C. tepidum, the RLP is involved in sulfur metabolism pathways other than MTA salvage (29). No change in ethylene production was observed in the MTA-grown R. palustris Δopl2 strain compared with wild type (Fig. 3B, purple), consistent with a lack of function of this gene in MTA metabolism. Last, to verify that the changes in ethylene production between various deletion strains were not due to pleiotropic effects on sulfur uptake, we measured the amount of sulfur source consumed by each strain (SI Appendix, Fig. S5 A and C). Other than i-methionine consumption in the ΔmtnP and Δrpl1 deletion strains, i-methionine and MTA consumption was similar in each strain, in further support that ethylene production was due to the specific function of each gene product. Taken together, these results demonstrated that ethylene production is directly linked to MTA metabolism and this process requires the activity of MtnP, MtnA, and Ald2. Furthermore, the enhanced ethylene production by R. rubrum and R. palustris Δrpl1 and Δcupin strains suggests that both the MTA-isoprenoid shunt and an ethylene-forming MSP are simultaneously functioning anaerobically.

Ald2 Functions as a 5-(Methylthio)ribulose-1-P Aldolase. Previous aerobic and anaerobic studies of MTA metabolism in R. rubrum established that gene products of mtnP and mtnA function as a bona fide MTA phosphorylase and 5-(methylthio)ribulose-1-P

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**Table 1. Total ethylene produced by R. rubrum grown on 500 μM MTA and expressing ald2 genes from various organisms**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Expressed ald2 gene (locus tag)*</th>
<th>Doubling time, h¹</th>
<th>Ethylene, μmol/L/OD₆₆₀ nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δrpl1</td>
<td>R. rubrum (Rru_A0359)</td>
<td>24 ± 8</td>
<td>37 ± 6</td>
</tr>
<tr>
<td>Δrpl1/Δald2</td>
<td>None: no plasmid</td>
<td>39 ± 9</td>
<td>0.50 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>None: empty plasmid</td>
<td>40 ± 4</td>
<td>1.01 ± 0.72</td>
</tr>
<tr>
<td>R. rubrum (Rru_A0359)</td>
<td>17 ± 7</td>
<td></td>
<td>29.2 ± 7.1</td>
</tr>
<tr>
<td>R. palustris (RPA4655)</td>
<td>26 ± 3</td>
<td></td>
<td>67.9 ± 12.1</td>
</tr>
<tr>
<td>M. morganii (MU9_3121)</td>
<td>26 ± 5</td>
<td></td>
<td>56.2 ± 5.7</td>
</tr>
<tr>
<td>E. limasum (Eli_0530)</td>
<td>15 ± 4</td>
<td></td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>E. coli (C201_13072)</td>
<td>28 ± 5</td>
<td></td>
<td>43.0 ± 7.0</td>
</tr>
</tbody>
</table>

*For R. rubrum Δrpl1 strain, the ald2 gene was expressed from its native location on the chromosomes; for Δrpl1/Δald2 strain, genes were expressed from pMTAP-MCS3-based plasmids listed in SI Appendix, Table S1.

¹Growth doubling time determined by fit of growth data to sigmoidal-logistic function (24) for n = 3 independent growth experiments.

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**Fig. 3.** Ethylene production in the presence of various sulfur sources. (A and B) Total ethylene produced [micromoles of ethylene per liter culture per optical density measured at 660 nm (μmol/L/OD₆₆₀ nm)] by R. rubrum and R. palustris, respectively, when grown to stationary phase on L-methionine (Met), MTA, or 2-(methylthio)ethanol (MT-EtOH) supplied at 1 mM. (C and D) R. rubrum ethylene induction after switching to anaerobic growth on 2-(methylthio)ethanol. Cultures were initially grown aerobically on 1 mM ammonium sulfate (triangles), anaerobically on 1 mM ammonium sulfate (circles), or anaerobically on 1 mM 2-(methylthio)ethanol (squares) before being washed into anaerobic media containing 1 mM 2-(methylthio)ethanol without or with 15 μg/mL chloramphenicol. Error bars are SDs from n = 3 independent induction experiments. Dashed lines are fits of data to a sigmoidal-logistic model to determine onset of ethylene production given by when ethylene exceeds the 0.01 μmol/L/OD₆₆₀ nm detection limit.
isomerase, respectively, to form 5-(methylthio)ribulose-1-P from MTA (16, 17) (Fig. 1, enzymes D and E). These studies also indicated that the \textit{R. rubrum ald2} gene product could function aerobically in vitro to cleave 5-(methylthio)ribulose-1-P into dihydroxyacetone-phosphate and by inference 2-(methylthio)acetaldehyde (16) (Fig. 1, compound 14). Given that our complementation data suggested that the \textit{ald2} gene product functioned in vivo in anaerobic MTA metabolism and ethylene production in both organisms, we sought to determine the anaerobic function of Ald2. The recombinant \textit{R. rubrum} putative aldolase protein was synthesized in \textit{E. coli} and purified by anaerobic His, affinity chromatography. With anaerobic in vitro assays, 5-(methylthio)ribulose-1-P was shown to serve as a substrate for \textit{R. rubrum} Ald2 to yield 2-(methylthio)acetaldehyde as determined by GC analysis using known standards (Fig. 4A). Identity was further confirmed via reduction to 2-(methylthio) ethanol by \textit{Saccharomyces cerevisiae} alcohol dehydrogenase (yeast ADH) (Fig. 4B). 5-(Methylthio)ribulose-1-P did not serve as a substrate for Ald2. However, the addition of purified \textit{R. rubrum} MtnA to 5-(methylthio)ribulose-1-P resulted in the production of 5-(methylthio)ribulose-1-P, which was subsequently converted to 2-(methylthio)acetaldehyde by Ald2 (Fig. 4A and B). Together, these results demonstrated that Ald2 functions as a 5-(methylthio)ribulose-1-P aldolase to yield 2-(methylthio)acetaldehyde and dihydroxyacetone phosphate (16) as the products.

To ascertain the in vivo function of Ald2 as a 5-(methylthio)ribulose-1-P aldolase, we performed [methyl-\textsuperscript{14}C]MTA feedings of \textit{R. rubrum} RLP deletion strain (\textit{Δrlp1}), since this strain shows the highest levels of ethylene production from growth on MTA. \textsuperscript{14}C-labeled intermediates produced by MTA metabolism were resolved by reverse-phase HPLC and identified by in-line radiometric flow detection based on known standards. 2-(Methylthio)acetaldehyde was not observed in feeding experiments (R\textsubscript{T} = 18.1 min), presumably due to rapid conversion in vivo to a less reactive species (Fig. 4C). Rather, an unknown peak was transiently observed with R\textsubscript{T} = 20.1 min, which was subsequently identified as 2-(methylthio)ethanol (Fig. 1, compound 15). Moreover, this peak was absent in feeding experiments of \textit{R. rubrum} strain \textit{Δrlp1/Δald2} in which the aldolase was inactivated (Fig. 4D). Additionally, the requirement of MTA phosphorylase and 5-(methylthio)ribulose-1-P isomerase activity for metabolism of MTA to 2-(methylthio)ethanol was likewise observed in feedings of \textit{R. rubrum} strains \textit{ΔmtnP} and \textit{ΔmtnA}, respectively (Fig. 4E). 2-(Methylthio)ethanol was similarly identified in feedings of \textit{R. palustris} (SI Appendix, Fig. S6), although at lower levels due to slower MTA uptake and metabolism by \textit{R. rubrum}. Altogether, this is consistent with the conclusion that in vivo Ald2 acts upon 5-(methylthio)ribulose-1-P to form 2-(methylthio)acetaldehyde, which is then reduced to 2-(methylthio)ethanol (Fig. 1, brown arrows). The ability of commercial yeast ADH to catalyze this reduction in vitro suggested a similar

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** Identification of reaction catalyzed by Ald2. (A) GC traces identifying specific conversion of 5-(methylthio)ribulose-1-P to 2-(methylthio)acetaldehyde catalyzed by purified \textit{R. rubrum} Ald2 protein. Conversion of 5-(methylthio)ribulose-1-P to 5-(methylthio)ribulose-1-P isomerase protein (MtnA). (B) GC traces identifying specific conversion of 2-(methylthio)acetaldehyde to 2-(methylthio)ethanol by yeast ADH. (C and D) [methyl-\textsuperscript{14}C]-Metabolites produced by \textit{R. rubrum} strains \textit{Δrlp1} and \textit{Δrlp1/Δald2}, respectively, at the indicated time (h) after feeding [methyl-\textsuperscript{14}C]MTA. (E) [methyl-\textsuperscript{14}C]2-(Methylthio)ethanol observed in the indicated \textit{R. rubrum} strain at the indicated time after feeding [methyl-\textsuperscript{14}C]MTA. (F) Ethylene stoichiometry measurements from 2-(methylthio)ethanol. Total ethylene produced (in nanomoles) from the indicated amount of 2-(methylthio)ethanol (in nanomoles) fed to \textit{R. rubrum} (circles) or \textit{R. palustris} (squares). Error bars are SD from three independent feedings. Data were fit to a linear regression. Compounds: (i) 5-(methylthio)ribulose-1-P (MTRu-1P), (ii) 5-(methylthio)ribulose-1-P (MTR-1P), (iii) 2-(methylthio)acetaldehyde (MT-adh), (iv) 5-(methylthio)ethanol (MT-ETHOH), (v) 5-(methylthio)ribulose, (vi) 5-(methylthio)ribulose, (vii) unknown not involved in anaerobic ethylene-forming MSP, and (viii) unknown contaminant present in commercial [methyl-\textsuperscript{14}C]SAM.
ADH reduction may be occurring in *R. rubrum* and *R. palustris*. However, which, if any, of the multiple ADH-like enzymes encoded by these organisms’ genomes is catalyzing this reaction is currently unknown.

2-(Methylthio)ethanol Is an MSP Intermediate and Likely Anaerobic Precursor to Ethylene. The in vivo dependence upon *mtnP*, *mtnA*, and *ald2* gene products for synthesis of 2-(methylthio)ethanol and ethanol from MTA suggested a link between 2-(methylthio)ethanol metabolism and ethylene synthesis. Unfortunately, no additional 14C-labeled metabolites produced from subsequent metabolism of 2-(methylthio)ethanol were resolved during MTA feedings. Therefore, *R. rubrum* and *R. palustris* strains were cultured anaerobically with 2-(methylthio)ethanol as sole sulfur source, and their ability to grow and produce ethylene was quantified. All strains equally grew on 2-(methylthio)ethanol as sole sulfur source (*SI Appendix*, Fig. S1). All strains, including those compromised for ethylene production from MTA, produced a total of ~100 μmol/L OD₆₅₀nm of ethylene during growth on 2-(methylthio)ethanol, save for the *R. palustris ΔmtnP* strain (Fig. 3 A and B). Given that ethylene is a regulatory hormone for growth and stress response in plants and some bacteria (30–32), one possibility for this consistent 100 μmol/L OD₆₅₀nm production is that ethylene accumulation to such high levels in the culture headspace is inhibitory to further production. However, it was found that total ethylene production was independent of the headspace ethylene concentration, indicating that ethylene was not inhibitory at these levels to *R. rubrum* and *R. palustris* (*SI Appendix*, Fig. S7).

Subsequent analysis of 2-(methylthio)ethanol consumption during growth revealed that all strains consumed ~100–150 μmol/L OD₆₅₀nm of 2-(methylthio)ethanol (*SI Appendix*, Fig. S5 A and C). This indicated that, for nearly every mole of 2-(methylthio)ethanol consumed, 1 mol of ethylene was produced (*SI Appendix*, Fig. S5 B and D). To more accurately quantify the stoichiometry of this process, cells were grown on 2-(methylthio)ethanol to midexponential phase, washed into sulfur-free media, and then fed with varying amounts of 2-(methylthio)ethanol. For each mole supplied, an equivalent mole of ethylene was produced in both organisms (Fig. 4F), confirming a 1:1 stoichiometry. These observations further confirm that 2-(methylthio)ethanol is an intermediate in the anaerobic ethylene-forming MSP, and indicate that 2-(methylthio)ethanol or an unidentified cosubstrate is the likely precursor to ethylene.

Substrate specificity for this process appears limited to 2-(methylthio)acetaldehyde and 2-(methylthio)ethanol. Similar methylthio-containing substrates were tested for growth and ethylene production by *R. rubrum* (*SI Appendix*, Table S4). Neither the methionine degradation pathway intermediates 2-keto-4-(methylthio) butyric acid, methional, and methionol; nor the amino acids l-cysteine and S-methyl-l-cysteine; nor the 2-(methylthio)ethanol analogs 2-(methylthio)acetate and 2-mercaptoethanol led to production of ethylene above background levels, indicating that ethylene was not inhibitory at these levels to *R. rubrum* and *R. palustris* (*SI Appendix*, Fig. S7). When washed into sulfur-free media, and then fed with varying amounts of 2-(methylthio)ethanol, ethylene analogs 2-(methylthio)acetaldehyde and 2-(methylthio)ethanol were tested for growth and ethylene production by *R. rubrum* and *R. palustris* (**Table S5**). Complementation with *mtnA* as part of a putative anaerobic MSP was identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) sequence similarity network. Organisms possessing *mtnP*, *mtnK*, or *mtnA* homologs as part of the universal MSP and organisms possessing *ald2* homologs as part of fuculose metabolism were filtered from the dataset. Of the 3,914 bacterial genomes annotated in the KEGG database, 256 organisms (6.5%) contained these genes either as a single gene cluster (131, 3.3%) as in the case of *R. rubrum*, with two genes together and the third elsewhere (112, 2.9%) as in the case of *R. palustris* (Fig. 2A and **SI Appendix**, Table S5). In a few species (13, 0.3%), mostly from the aerobic methylotroph family, all three genes were located separately, suggesting a different function in such organisms. These 256 representative organisms were composed of *Proteobacteria* (α, 28%; β, 10%; γ, 8%; ε, 4%), *Chloroflexi/Cytophacter* (6%), *Bacilli* (18%), and *Cyanobacteria* (22%), suggesting widespread occurrence of this MSP among bacteria (*SI Appendix*, Table S5). Consistent with a putative role for these genes in anaerobic MTA metabolism, 36% of the identified organisms were known obligate anaerobes, 46% were facultative anaerobes, 5% were of unknown physiology, and only 12% were known obligate aerobes (*SI Appendix*, Table S5). Additionally, known habitats of these organisms were predominantly terrestrial. Soil and soil sediment bacteria composed 43% of the organisms, a third of which (33/111) were known endophytes particularly from the root nodulating rhizobia family. Other terrestrial bacteria were identified in freshwater and groundwater environments (12%); niche environments such as hot springs, acid/alkaline/hypersaline lakes (7%); anthropically contaminated soil and freshwater environments (15%); and as pathogens, commensals, or symbionts of nonplant terrestrial organisms (14%). Only 8% of the identified species were from marine environments (*SI Appendix*, Table S5). Currently, beyond *R. rubrum* and *R. palustris*, it is unknown whether any of these organisms can perform anaerobic MTA metabolism. To ascertain whether homologous *ald2* genes identified in other organisms in conjunction with *mtnP* (or *mtnK*) and *mtnA* as part of a putative anaerobic MSP could function as a 5-(methylthio)ribulose-1-P aldolase, we complemented the *R. rubrum Δrlp1 Δald2* strain with *ald2* genes from representative organisms (Fig. 2A and Table 1). Complementation with *ald2* from the opportunistic pathogen, *Morganella morganii* (γ-proteobacteria), and the commensal obligate anaerobe, *Eubacterium limosum* (clostridia) also restored ethylene production when grown using MTA as sole sulfur source, albeit to a lesser extent
with the *E. limosum* enzyme, suggesting a similar role for these putative *ald2* genes in their respective organisms. Thus far, attempts to formulate a defined growth media for testing whether *M. morganii* and *E. limosum* can grow and metabolize MTA were unsuccessful. Given the strong amino acid sequence homology between the *R. rubrum* Ald2 and *E. coli* FucA (52% identity; E value, 4e−65), we also complemented the *R. rubrum* ΔfucA/Δald2 strain with *fucA* from *E. coli* S17. Ethylene production was restored by *fucA* to the same levels observed when complemented with the *R. rubrum* ald2 gene (Table 1). As *FucA* is a class II aldolase and in the synthesis direction accepts a wide variety of aldehydes substrates for aldol condensation with dihydroxyacetone phosphate (33), this suggests that the *R. rubrum* Ald2 and FucA enzymes likely share a similar reaction mechanism.

### Discussion

Sulfur (S) metabolism is an essential metabolic process for all organisms, and on average, bacteria contain ~1−2% organic sulfur by dry cell weight (34). While bacteria such as the enterics and those inhabiting marine ecosystems, salt marshes, and volcanic thermal vents live in S-rich environments (35−37), many other bacteria are faced with the challenge of limited S availability. The average total S in freshwater ecosystems is ~100 μM, typically in the form of sulfate (38). Lake and river water ranges from 0.065 to 100 μM sulfate (39, 40), and wetlands and low-salinity marshes contain 100−3,000 μM total S (41−43). While soils and sediments are typically also rich in S (3−400 mmol/kg) (44, 45), pore water in anoxic bog soils can contain as low as 20 μM S (46, 47), similar to rainwater, and anoxic soil pore water in flooded soils can vary greatly from 20 μM to 20 mM total S (48, 49). Thus, bacteria have developed multiple mechanisms for coping with S limitation. Growth situations where there is a high S demand (e.g., cell division, sporulation, metabolic shifts) or where S sources are poor or limiting result in an observed up-regulation of numerous sulfur metabolism-related proteins. These include inorganic and organic S transporters for scavenging sulfur, l-methionine and l-cysteine synthesis enzymes, and sulfur salvage proteins involved in the active methyl cycle, the universal MSP, and the MTA-isoprenoid shunt (7, 17, 21, 50, 51). The anaerobic ethylene-forming MSP described herein not only metabolizes MTA into a usable organic sulfur metabolite, presumably methionine, but also leads to the production of ethylene. Liberated ethylene does not appear to be consumed and further metabolized by *R. rubrum* or *R. palustris* as evidenced by the stoichiometry measurements (Fig. 4F). Moreover, concentrations of at least 500 μM ethylene does not appear to inhibit growth of these organisms, and any additional regulatory effects of ethylene upon these organisms have yet to be observed. In the environment, ethylene is an essential plant hormone that stimulates vegetative growth, fruit ripening, and flower opening; inhibits root formation; and induces leaf senescence and abscission (30, 31, 52). Ethylene also functions in a wide array of plant interactions with soil-borne microbes (53, 54). Of the identified organisms, 43% are common inhabitants of soil and 33 of these species are known endophytes particularly from the root-nodulating, nitrogen-fixing rhizobia family.

Multiple pathways have been discovered for biotic ethylene metabolism, both enzymatic and nonenzymatic. Currently known enzymatic routes to ethylene are the ethylene-forming enzyme (EFE) and ACC pathways, both of which are obligately aerobic processes (Fig. 5 A and C). EFE from bacteria and fungi such as *Pseudomonas syringae* and *Aspergillus nidulans*, respectively, utilize α-ketoglutarate and dioxygen to generate ethylene (55). In plants, ethylene is produced primarily from SAM via ACC synthase and ACC oxidase (56). In many organisms, ethylene is produced at low levels by nonenzymatic flavin-mediated photoperoxidation or hydroxyl radical attack of l-methionine degradation pathway products (57, 58) (Fig. 5B). In *R. rubrum*, l-methionine generated by the ethylene-forming MSP could be degraded and then nonenzymatically oxidized to produce the observed ethylene.

### Table 2. Maximum specific rate of ethylene production achieved during anaerobic growth with the indicated sulfur source

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sulfur source</th>
<th>Ethylene rate, * μmol/L/h/OD₆₆₀ nm</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. rubrum</em> wild type</td>
<td>1,000 μM SO₄²⁻</td>
<td>7.2 ± 3.2 × 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>100 μM SO₄²⁻</td>
<td>3.0 ± 1.0 × 10⁻²</td>
</tr>
<tr>
<td></td>
<td>1,000 μM MT-ETHOH¹</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td><em>R. palustris</em> wild type</td>
<td>1,000 μM SO₄²⁻</td>
<td>&lt;2 × 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>100 μM SO₄²⁻</td>
<td>8.2 ± 1.5 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>1,000 μM MT-ETHOH¹</td>
<td>7.2 ± 1.1</td>
</tr>
</tbody>
</table>

*Specific rate measured in micromoles of ethylene per liter culture per hour per optical density measured at 660 nm (μmol/L/OD₆₆₀ nm) for n = 3 independent growth experiments. Rate was calculated by measuring the ethylene produced (μmol/L/OD₆₆₀ nm) at 30-min intervals during early-exponential, midexponential, late-exponential, and stationary phases.

¹ MT-ETHOH, 2-(methylthio)ethanol.

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PNAS | Published online November 13, 2017 | E10461
However, this does not appear to be the case as L-methionine, 2-keto-4-(methylthio)butyrate, methionol supplied as the sole S source produced substantially less ethylene than MTA or 2-(methylthio)ethanol (SI Appendix, Table S4). Rather, a specific process involving 2-(methylthio)ethanol that requires protein synthesis appears to be operating as indicated by the induction and stoichiometry experiments (Fig. 3 C and D). The results support two possible models for the remaining unknown steps of 2-(methylthio)ethanol metabolism and concurrent ethylene production. Given that free methanethiol is synthesized only via the MTA-isoprenoid shunt (16, 24), 2-(methylthio)ethanol is likely not simply cleaved into free methanethiol and ethylene or a precursor thereof. Rather, either (i) the methylthio group of 2-(methylthio)ethanol is transferred to an unknown cosubstrate to form a metabolizable organic sulfur compound and ethylene arises from the 2-(methylthio)ethanol backbone or (ii) an unknown cosubstrate and 2-(methylthio) ethanol are condensed together and ethylene arises from the unknown cosubstrate (Fig. 1, brown dashed lines).

Biological anaerobic ethylene production has long been observed in anoxic environments such as waterlogged soils (59, 60). Ethylene levels of up to ~20 ppm (715 μmol/kg soil) routinely accumulate in anoxic soils within 3–10 d of waterlogging, and these levels are considerably higher than that required to inhibit plant root growth. Moreover, the process of ethylene production appears to be primarily enzymatic in nature and ethylene is not observed until oxygen levels drop below 2%, indicating that the EFE and ACC pathways (Fig. 5 A and C) are not the main mechanisms. There is conflicting evidence as to whether bacteria or fungi are primarily responsible for anoxic ethylene production, and the mechanisms are largely unknown beyond a link to methionine metabolism. Treatment and analysis of waterlogged soils suggest that bacteria may be the primary source, but to date all ethylene-producing bacteria isolated by L-methionine enrichments only exhibit aerobic ethylene production (61, 62).

Similarly, the dominant soil fungi, Mucor hiemalis, proposed to be the primary ethylene producer, also requires oxygen and L-methionine to synthesize ethylene (63, 64).

Given that this exclusively anaerobic MSP demonstrated in this study links MTA metabolism to ethylene production, MTA may be the precursor to ethylene generated by anoxic soil environments. This was supported by the observation that organisms such as R. rubrum, R. palustris, Rhizobia sp., Bacillus cereus, and Bacillus thuringiensis are widely distributed soil and water facultative anaerobes and potentially possess a functional anaerobic ethylene-forming MSP (Fig. 2 A and SI Appendix, Table S5). MTA as a precursor to anaerobic ethylene may arise from cellular processes within these organisms or supplied extracellularly, excreted by organisms lacking a functional anaerobic MSP (12). Extracellularly acquired 2-(methylthio)acetaldehyde and 2-(methylthio)ethanol may also be a contributor to anaerobic ethylene production. In organisms such as Lactococcus sp., 2-(methylthio)acetaldehyde arises from chemical degradation of L-methionine and 2-keto-4-(methylthio)butyrate (65), and 2-(methylthio)acetaldehyde and 2-(methylthio)ethanol have also been identified at high levels (~350 μg/kg) in tomato (66). Together with the observation that the ethylene-forming MSP is exclusively anaerobic, this is consistent with the conclusions of Lynch and Harper (64) that soil anaerobiosis probably mobilizes the substrates required for biological ethylene formation. It will be of interest to enrich for and isolate other organisms from the environment capable of anaerobic methionine salvage and ethylene production using organic sulfur substrates other than methionine. Indeed, in organisms bearing functional MtnP, MtnA, and Ald2 enzymes to convert MTA to 2-(methylthio)acetaldehyde, subsequent metabolism of 2-(methylthio)acetaldehyde may proceed via a route different from ethylene production to regenerate methionine. Regardless, the fact that putative mtnP, mtnA, and ald2 genes are widely distributed among bacteria, particularly facultative and obligate anaerobes,
suggests that anaerobic methionine salvage may be a common mechanism for maintaining appropriate intracellular S pools in sulfur-poor environments.

Materials and Methods

Chemicals and Helper Enzymes. Ammonium sulfate, L-methionine, L-cysteine, S-methyl-L-cysteine, 5’-methylthioadenosine, 2-keto-4-(methylthio)butyric acid, 2-mercaptoethanol, and NADH were all from Sigma-Aldrich. 3-(Methylthio)propan-1-ol (methionol), 2-(methylthio)ethanol, 2-(methylthio)acetaldehyde dimethylacetal, and 2-(methylthio)acetate were all from Alfa-Aesar. Synthesis of 2-(methylthio)acetaldehyde from the dimethylosalicylic form was performed by combining 90 µL of 2-(methylthio)acetaldehyde-dimethylacetal with 200 µL of 1% HCl in water in a 1-mL sealed vial under argon. The reaction was boiled in a hot water bath for 30 min and subsequently verified to have proceeded to completion by reverse-phase HPLC on a C18 column (Agilent) using a gradient of 0.1–25% acetonitrile in 20 mM ammonium acetate, pH 6.8, over 25 min. The resulting 4.5 M 2-(methylthio)acetaldehyde was immediately used for growth studies or enzymatic assays.

R. rubrum MTA phosphorylase (MtnP), B. subtilis MTR kinase (MtnK) and MTR-1P isomerase (MtnA) from both organisms were purified as previously described (16, 22). R. rubrum putative aldolase (Alld2) recombining protein synthesis (16) was modified as detailed in SI Appendix. Saccharomyces cerevisiae alcohol dehydrogenase (yeast ADH) was from Sigma. All restriction endonucleases, T4 DNA ligase, and Phusion DNA polymerase for PCR were from New England Biolabs. All genomic DNA for PCR amplification was isolated from the respective organisms using a DNeasy extraction kit (Qiagen).

Stoichiometry Measurements. Five hundred milliliters of wild-type R. rubrum or R. palustris cultures were grown to late-exponential phase (OD660 nm ~1.0) with 1 mM 2-(methylthio)ethanol as sole sulfur source. Cells were washed three times anaerobically with sulfur-free media, and then centrifuged, washed anaerobically three times into sulfur-free media, and used to inoculate fresh anaerobic media supplemented with the indicated sulfur source as detailed in SI Appendix.

Characterization of R. rubrum Ald2 Protein. For enzymatic assays, all components were allowed to equilibrate on ice for 1 h in an anaerobic chamber before initiating the reactions. 5-(Methylthio)ribose-1-P or 5-(methylthio)ribulose-1-P substrates were added at 20 µM final concentration to reaction buffer containing 5 mM MgCl2, 25 mM Mops-KOH, pH 7.5, and 20 µM purified R. rubrum Ald2 in a 125-µL total volume. Purified R. rubrum MtnA was added to 20 µM final concentration when 5-(methylthio)ribose-1-P was the starting substrate. Reactions were incubated at 30 °C for 2 h. Products were verified by GC on a Shimadzu GC-14A with flame ionization detector. One microliter of sample was injected onto an Agilent DB-wax column with helium carrier gas at 40 °C for 2 min followed by a temperature gradient of 0.1 °C/min to 180 °C. Yeast ADH catalyzed the reduction of 2-(methylthio)acetaldehyde generated by Ald2 to 2-(methylthio)ethanol after combining 100 µL of the above completed reactions with 0.05 mg/mL yeast ADH and 1 mM β-NAD+ (Sigma). Reactions were incubated for 2 h at 30 °C, and then analyzed by GC as detailed above. Yeast ADH was prepared by dissolving lyophilized enzyme into anaerobic 10 mM sodium phosphate buffer, pH 7.5, at 4 °C to 1 mg/mL final concentration. β-NAD+ was prepared by dissolving it into anaerobic water at 4 °C to 10 mM final concentration.

Induction Experiments of Ethylene Production. The R. rubrum wild-type strain was initially grown anaerobically (20 mL in 28-mL culture tubes) or aerobically (20 mL in 50-mL flask) with 1 mM ammonium sulfate or 2-(methylthio)ethanol as sole sulfur source to late-exponential phase (OD600 nm ~1.0). Note that R. rubrum could not grow aerobically on 2-(methylthio)ethanol. Cells were washed anaerobically three times with sulfur-free media, and then resuspended to a final OD600 nm of ~0.5 in sulfur free media. Ten milliliters of culture were aliquoted into 25-mL anaerobic vials, and the indicated amount of 2-(methylthio)ethanol was added. Cultures were further incubated at 30 °C under illumination for 12 h, then ethylene was quantified in the headspace as detailed above.

ACKNOWLEDGMENTS. We thank the Ohio State University Campus Chemical Instrument Center and Jeremy Keasler for providing use of Thermo Scientific DSQ-1 GC-MS and for help with data collection. This work was initially supported by NIH Grant GM095742 (to F.R.T.) and by a Ruth L. Kirshstein National Research Service Award from the National Institute of General Medical Sciences (F32GM109547) to J.A.N.
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