Anaerobic arsenic methylation as a microbial warfare strategy

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ABSTRACT

Microbial arsenic methylation is established as a detoxification process under aerobic conditions (converting arsenite to monomethylated arsenate) but proposed to be a microbial warfare strategy under anoxic conditions due to the toxicity of its main product monomethylarsonous acid (MMAs(III)). Here we leveraged a paddy soil-derived anaerobic arsenic methylator, *Paraclostridium bifermentans* strain EML to gain insights into this process. Strain EML was inoculated into a series of media involving systematic dilutions of Reinforced Clostridial Broth (RCB) with 25 μM arsenite to assess the impact of growth substrate on arsenic methylation. Concentrations of MMAs(III) and *arsM* gene transcription were found to be positively correlated with the RCB dilution, suggesting that substrate limitation enhances *arsM* gene expression and associated anaerobic arsenic methylation. Anaerobic co-cultures of strain EML with either wild-type *Escherichia coli* K-12 MG1655 (WT) or *E. coli* expressing the MMAs(III)-resistance gene (*arsP*), ArsP *E. coli*, evidenced increased MMAs(III) production in the presence of *E. coli* than its absence and growth inhibition of WT *E. coli* to a greater extent than ArsP *E. coli*, presumably due to MMAs(III) produced by strain EML. Our findings point to an ecological role for anaerobic arsenic methylation, providing support for a microbial warfare function for this process.

Key words: anaerobic arsenic methylation, MMAs(III), *arsM* gene transcript, anaerobic co-culture, microbial warfare, *arsP*, *E. coli* MG1655

SYNOPSIS: Understanding the controls on anaerobic microbial arsenic methylation is relevant for rice paddy soils and helps to ensure food security.

INTRODUCTION

Microbial transformations play an important role in the biogeochemical cycling of arsenic (As) in the environment, and include reduction, oxidation, thiolation, methylation, and demethylation of inorganic and organic As.[1-4] These reactions impact the mobility, bioavailability, and toxicity of As compounds.[1-3, 5, 6] In recent years, microbial transformations of As in paddy soil have drawn increasing attention because of the potential health risk of dietary exposure of As from rice-containing products.[7, 8] For instance, organic As (in particular dimethyl arsenate, DMAs(V)) is commonly detected in rice grains, along with inorganic As.[9, 10] As DMAs(V) is much less toxic than arsenite, accumulation of DMAs(V) in rice grains largely reduces its toxicity to humans. However, there is evidence of a correlation between DMAs(V) accumulation in rice grains and rice straight-head disease, a condition that decreases crop yield.[11, 12]

Arsenic methylation is a microbially-mediated process involving the transformation of inorganic trivalent As (iAs(III)) into mono-, di-, and trimethylated As compounds and is catalyzed by *S*-adenosyl-methionine methyltransferase (ArsM in prokaryotes).[13-15] Generally, As methylation occurring under oxic conditions is proposed as an iAs(III)-detoxifying process because although more toxic As compounds (monomethylarsonous acid (MMAs(III) and dimethylarsinous acid (DMAs(III)) are produced, they are rapidly oxidized in the presence of O_2 to their less toxic pentavalent counterparts (monomethylarsonic acid (MMAs(V) and dimethylarsinic acid (DMAs(V)).[15] This paradigm is supported by the fact that the heterologous expression of the *arsM* gene conferred As(III) resistance to an As(III)-sensitive *Escherichia coli* strain under aerobic conditions.[15, 16]

In contrast, detoxification is unlikely to be the ecological function of As-methylators inhabiting anoxic environments since organic As products are present in their trivalent

forms. Interestingly, the evolutionary history of the *arsM* gene predicts its emergence during the anoxic Archaean era, when MMAs(III) would have been chemically stable.[17, 18] This finding suggests that the original function of MMAs(III) could have been to serve as a primitive antibiotic.[19, 20] At present, there is only one confirmed and available anaerobic As-methylating microorganism, *Paraclostridium bifermentans* strain EML (henceforth strain EML), a fermenter isolated from a paddy soil in Vietnam.[21] However, controls over its methylating activity remain poorly understood.

In microbial communities, competitive phenotypes (including production of antibiotics) can arise as a consequence of limited resources (e.g., nutrients, space).[22] Soils represent an ecosystem in which a large number of microorganisms compete for scarce resources, thus competition is widespread.[23] We hypothesize that competition for resources may boost As methylation by strain EML, conferring it an advantage over its competitors. In this study, we aim to test the microbial warfare hypothesis for anaerobic As methylation by investigating the effect of growth substrate limitation on As methylation by strain EML. Further, we probe direct microbial interaction/inhibition between the strain EML and potential competitors (*Escherichia coli* MG1655 either the wild-type strain (WT) or one engineered to express the MMAs(III)-resistance gene (*arsP*)). From an ecological point of view, it is reasonable to expect enhanced As methylation under substrate-limiting conditions as a response to resource competition. According to our hypothesis, strain EML would increase the production of toxic MMAs(III) under growth substrate-limited conditions in order to thwart other microorganisms competing for the same resources. Although DMAs(III) might also function as an antibiotic under anaerobic condition, we focus on MMAs(III) in this study due to the analytical limitations associated with DMAs(III).

MATERIALS AND METHODS

Growth Experiment. The anaerobic As-methylating bacterium, *Paraclostridium bifermentans* strain EML (henceforth strain EML) was previously isolated from an anaerobic paddy soil enrichment.[21, 24] To investigate how growth substrate availability affects As methylation activity, dilutions (v/v) of Reinforced Clostridial Broth (RCB) (Oxoid Ltd) medium in Milli-Q water (100% RCB, 75% RCB, 50% RCB, and 25% RCB) were prepared in 120 mL serum bottles containing 50 mL medium (Supporting information Table S1). The medium was brought to a boil for 5 min to remove O_2, then cooled down under a 100% N_2 gas flow to room temperature, and dispensed into individual culture serum bottle under the same N_2 atmosphere. The bottles were then sealed with sterile rubber stoppers and crimped with aluminum caps and the headspace was flushed with 100% N_2 to ensure anaerobic conditions before autoclaving at 121°C for 15 mins.

A pre-culture of strain EML was grown in RCB anaerobically to mid-exponential growth phase. Strain EML (~0.5 mL) was inoculated into each RCB dilution containing 25 μM iAs(III) as sodium arsenite (or into the equivalent no-iAs(III) control) in triplicate using sterile N_2-flushed syringes and needles. The inoculum represents approximately 1% of the total volume (v/v). All the bottles were incubated at 30°C in the dark without shaking. A total of eight experimental conditions were selected (Table S1). At selected time points and for each condition, triplicate bottles were sampled for growth, which was quantified using both optical density at 600 nm (OD_{600}) and total protein content estimated using a BCA protein assay kit (Thermo Scientific, MA, USA). For quantification of the expression of the *arsM* gene, triplicate cultures were sampled for RNA extraction at 8 h and 24 h. To test the stability of MMAs(III) in RCB medium, additional abiotic control experiments (including fresh/spent RCB medium

supplemented with MMAs(III), and chemical reaction between MMAs(III) and sulfide) were performed in duplicate (see Supporting Information Text Methods SM1).

Arsenic Speciation. At each time point and for each condition, aqueous and intracellular As speciation and the total As were characterized. Samples for aqueous As species and total As were obtained from 1 mL of culture collected with sterile, N_2-flushed syringes and needles, filtered through 0.22 μm cellulosic membrane filters, and stored in 1 mL 1% HNO_3 (≥ 69 %, Honeywell Fluka). Additionally, to analyze As species and total As post-sample oxidation, another 1 mL of culture was obtained as described above and oxidized by adding 10% (v/v) hydrogen peroxide (w/v) (H_2O_2, 30%, Reactolab SA) and was kept overnight in a 1% HNO_3 solution. For soluble intracellular As species, 1 mL of culture was collected, the cells were pelleted at 8,000 *g* for 5 min, and stored at -20°C until use. To release soluble intracellular As,[25] the cell pellets were lysed in a lysis buffer (0.1% Triton X-100, 0.1% SDS, 10 mM EDTA, and 1 mM Tris-HCl) at 95°C for 15 min by vortexing every 3 min. The lysed cell suspension was subsequently centrifuged at 8,000 *g* for 5 min, and the pellet was resuspended in 200 μL 1 x PBS buffer and used for protein determination as described above. The supernatant was filtered through 0.22 μm filters and reserved for As speciation and total As analysis.

Both aqueous and soluble intracellular As speciation were determined by high performance liquid chromatography and inductively coupled plasma mass spectrometry (HPLC-ICP-MS) on an Agilent 8900 ICP-QQQ instrument. A previously described anion exchange protocol using the step-gradient elution mode with an As Spec anion exchange fast column (50 mm x 4.0 mm, PrinCen, Guangzhou, China) was used.[21] Six As standards were available: MMAs(III) as methyldiiodoarsine (Santa Cruz Biotechnology Inc.), TMAs(V)O as trimethyl arsine oxide (Argus Chemicals Srl., Italy),

DMAs(V) as sodium dimethylarsinate (ABCR, Germany), MMAs(V) as monomethylarsonic acid (Chemservice, PA, USA), As(III) as sodium arsenite ($NaAsO_2$) (Sigma-Aldrich, MO, USA),and As(V) as sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$) (Sigma-Aldrich, MO, USA). In addition, monomethylmonothioarsonic acid (MMMTAs(V)) was synthesized as previously described (Text SM1).[26] Total aqueous and soluble intracellular As concentrations were measured using the same ICP-MS instrument in stand-alone mode.[21]

RNA extraction and RT-qPCR. After sampling, each culture was amended with RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) following the manufacturer's recommendations to stabilize RNA and prevent its degradation. The RNeasy Mini Kit (Qiagen) was used following the manufacturer’s instructions with an initial sample preparation protocol from the Qiagen RNAprotect bacteria reagent handbook. Protocol 5 (enzymatic lysis, proteinase K digestion, and mechanical disruption of bacteria) was employed for cell lysis prior to RNA purification. Genomic DNA digestion was completed during RNA purification using the RNase-Free DNase set (Qiagen). Reverse transcription was performed with QuantiTect Rev. Transcription Kit (Qiagen). Detailed information about designing a specific *arsM* gene primer set, optimizing the PCR amplification condition, and constructing an *arsM* plasmid to use for standard curve are described in Text SM2. The RT-qPCR was carried out in a Mic PCR system (Bio Molecular Systems, Mic) using SYBR Green Master Mix. The reactions (10 µL total volume) contained 5 µL of 2 × SensiFAST™ SYBR No-ROX Kit (Bioline, London, UK), 0.2 µM of each *arsM* gene primer, 2.5 µL of cDNA, and 1% (v/v) bovine serum albumin (BSA) (Sigma). A 10-fold dilution series containing 10^7-10^1 copies of strain EML *arsM* plasmid DNA was used to generate a standard curve. All samples were run in quadruplicates. A NRT (no-reverse transcriptase control) and a NTC (no template

control) were both included as negative controls.

Quantification of *arsM* gene transcripts. To get a comprehensive understanding of *arsM* gene expression under variable substrate conditions, both relative and absolute quantification methods were attempted. For relative quantification, the specific primer sets for the housekeeping genes considered and the corresponding amplification conditions are shown in Table S2. However, these genes are not used in this study because they do not meet the minimum requirement for a stable reference gene (see results in Section *arsM* gene transcription). For absolute quantification, we were concerned that the mRNA yield would be variable across conditions due both to biological reasons (e.g., rate and extent of growth) and biases introduced by RNA extraction. To obtain robust results, we compared two methods of absolute quantification. The first method entailed adjusting biomass for each culture prior to RNA extraction to ensure that RNA was extracted from the same amount of biomass (OD_{600}) regardless of conditions. The same volume of total RNA was used for reverse transcription, and the expression data (*arsM* copy numbers) were presented relative to OD_{600} (biomass). The second method consisted of quantifying extracted total RNA and using the same amount of RNA from all conditions in reverse transcription. The expression data were then normalized to the corresponding protein concentration.

Anaerobic co-culture system. To provide direct evidence of anaerobic As methylation resulting in microbial warfare, anaerobic co-culture systems (predator-prey systems) were established. The “predator” was strain EML.[21] One “prey” was wild type *Escherichia coli* K-12 strain MG1655 (WT), which is sensitive to MMAs(III) (Figures S1 and S2), and resistant to As(III)[27] (Figure S3 and Text SM3). The other was the same strain in which the *arsP* gene was integrated into the chromosome[28] and regulated by an oxygen-sensitive promoter *fnr*S (Texts SM4-SM6), a highly conserved, anaerobically

induced small RNA.[29] The *arsP* gene encodes ArsP, a MMAs(III) efflux permease that extrudes trivalent organoarsenicals from cells.[30] The expression of *arsP* in *E. coli* confers MMAs(III) resistance under anoxic conditions. The confirmation of As(III)-resistance in WT *E. coli* and *E. coli* expressing *arsP* (hereafter, ArsP *E. coli*), MMAs(III)-sensitivity in WT *E. coli*, and MMAs(III)-resistance in ArsP *E. coli* were provided in Figures S1-S3 and Supporting Information Text Results SR1 and SR2.

Co-culture treatments consisting of (i) strain EML + WT *E. coli* + 25 μM iAs(III), (ii) strain EML + ArsP *E. coli* + 25 μM iAs(III), or (iii) strain EML + 25 μM iAs(III) were conducted in triplicate as described above in anaerobic serum bottles containing 50 mL 100% RCB medium. Given the difference in growth rate between strain EML and *E. coli*, variable inoculation ratios between the co-culture members were tested and the optimal ratio found to be 10% EML (v/v), that is, the cell pellet from a 5 mL exponential phase culture of strain EML in RCB and 50 μL exponential phase culture of *E. coli* (WT or ArsP *E. coli*) in 50 mL of RCB (Figures S4-S6 and Texts SM7 and SR3). During the anaerobic co-culture period, aqueous As speciation, the growth rate, and *arsM* gene transcripts were measured by HPLC-ICP-MS, qPCR (by quantification of the 16S rRNA gene copy numbers of strain EML, and the two *E. coli* strains), and RT-qPCR, respectively.

RESULTS

iAs(III) inhibits strain EML growth. Strain EML was grown under anoxic conditions with various concentrations of RCB (100%, 75%, 50%, or 25% RCB) in the presence and absence of 25 μM iAs(III). The growth curves show that strain EML grew rapidly and reached the mid-exponential phase after about 8 hours in the absence of iAs(III) (Figure S7 and Tables S3 and S4). In contrast, the extent of growth of strain EML was

lower in the presence of iAs(III) (Figure S7 and Table S3) and it reached stationary phase sooner than in the absence of iAs(III), particularly in low growth-substrate media (50% and 25% RCB) (Figure S7 and Table S3). While strain EML harbors a gene encoding an iAs(III) efflux pump (*acr3*) (Figure S8), we hypothesize that it may not pump out intracellular iAs(III) sufficiently fast to preclude toxicity.

Impact of substrate concentration on MMAs(III) production by strain EML. To profile the dynamics of iAs(III) transformation during anaerobic growth of strain EML, time-dependent changes in As speciation in solution (aqueous) and inside cells (soluble intracellular) were monitored. Analysis of aqueous As species clearly shows that MMAs(III) was gradually produced by strain EML and increased during the exponential growth phase (0-12 hours) and reached a plateau between 24 and 48 hours (post-stationary to death growth phase) (Figures S9a-d and Table S5). Sterile RCB amended with 25 μM iAs(III) exhibited no transformation of iAs(III) (approximately 25 μM iAs(III) was detected at the beginning and end of incubation) (Table S6).

As expected, strain EML exhibited variable growth rates for varying RCB dilutions (Figure S10), confounding the interpretation of whether growth substrate levels affected the extent of As methylation. Normalization of methylated As (with and without oxidation) to protein concentration (Figure 1 and Table S7) reveals a trend in normalized MMAs(III) (Figure 1a) or MMAs(V)/DMAs(V) concentration as a function of RCB dilution (Figure 1b and 1c). Indeed, the normalized MMAs(III) concentration decreased in the following order: 25% RCB > 50% RCB > 75% RCB > 100% RCB (Figure 1). The greatest amount of protein-normalized MMAs(III) was produced by strain EML grown in the highest RCB dilution (25% RCB, 7,766 ± 919 nmol/g protein), which was about 14 (546 ± 39 nmol/g protein), 34 (224 ± 22 nmol/g protein), and 51 (150 ± 21 nmol/g protein) times higher than that in the 50%, 75%, and 100% RCB conditions, respectively (Figure

1a). Similar patterns were also observed for the oxidized samples, with the highest protein-normalized concentrations of MMAs(V) and DMAs(V) generated in the highest RCB dilution (25% RCB) (Figure 1b and 1c). Analysis of soluble intracellular As also clearly shows that strain EML accumulates high amounts of intracellular As(III) and MMAs(V) during anaerobic As methylation, particularly in the highest RCB dilution (25% RCB) (Figure S11 and Tables S8 and S9).

Chemical transformation of MMAs(III) in biological media. While MMAs(III) is clearly produced by strain EML, its chemical stability is often limited, even under anoxic conditions, due to side chemical reactions such as thiolation, resulting in an underestimation of the concentration of MMAs(III) produced. We tested this stability by amending anoxic RCB (100%-25%) or anoxic spent RCB (in which strain EML had grown) with 3 μM MMAs(III) and documented its significant disappearance from solution (after 24 h) (Figures S12 and S13 and Tables S10 and S11). Surprisingly, MMAs(III) stability was greatest in 100% RCB and lowest in 25% RCB, suggesting a negative correlation between MMAs(III) stability and medium dilution (Figures S12 and S13). Thus, the chemical stability of MMAs(III) in RCB medium (fresh or spent) is limited, suggesting that, in fact, MMAs(III) production is underestimated by our measurements in all conditions but more so in the more dilute RCB medium.

The disappearance of MMAs(III) from solution upon its amendment to RCB medium was puzzling and we hypothesized the formation of methylated-thiolated As species (e.g., monomethyldithioarsenate, MMDTAs(V)), some of which are not identifiable analytically in our system. To probe this possibility, we analyzed As speciation after oxidation of trivalent As species by 10% (v/v) H_2O_2.[31-33] Oxidation is expected to transform MMAs(III) into MMAs(V) quantitatively and to oxidize the thiol group in MMDTAs (and other monomethylated-thiolated species) to sulfate, which is

released, leaving MMAs(V) as the final product. Indeed, after oxidation, the concentration of MMAs(V) was greater than that of MMAs(III) measured prior to oxidation (Figure 1 and S9e-f and Text SR4 and Table S5), suggesting the oxidation of monomethylated As compounds other than MMAs(III) to MMAs(V).

Direct evidence of MMAs(III) chemical transformation was provided by the reaction of sulfide with MMAs(III) and subsequent retention of part of the As species by the column (Figure S14a). Furthermore, following oxidation with H_2O_2, the entire As inventory is recovered as MMAs(V) (Figure S14b and Table S12). Therefore, we proposed that compounds (such as MMDTAs(V) or others) are formed via the chemical reaction of MMAs(III) with reduced sulfur compounds in the growth medium. These reduced chemical species are likely retained by the HPLC column. If the samples are oxidized prior to measurement, the monomethylated-thiolated species are oxidized to MMAs(V), which is readily eluted. Thus, in oxidized samples, MMAs(V) corresponds to the sum of MMAs(III) and monomethylated-thiolated As species.

***arsM* gene transcription under variable substrate conditions.** In order to investigate whether the transcription of the gene responsible for As(III) methylation (*arsM*) responded to growth substrate concentration, gene expression was quantified using RT-qPCR. We first attempted to use relative expression analysis and evaluated the expression stability of 8 potential reference genes (Table S2) with the qBase plus software. Unfortunately, no optimal number of reference genes could be found due to the relatively high variability amongst sequential normalization factors (geNorm V > 0.15) and also only medium expression stability was achieved (0.5 < average geNorm M ≤ 1.0) (Figure S15). We presume that the considerable variation in growth rate due to RCB dilutions markedly impacted gene expression, even for so-called housekeeping genes. Therefore, we turned to absolute quantification. We adjusted the biomass

(OD_{600}) of strain EML obtained through variable RCB dilutions to approximately the same value prior to RNA extraction to eliminate possible biomass-related biases in RNA extraction and reverse transcription. As expected, the transcripts of strain EML *arsM* gene (adjusted to OD_{600}) were significantly higher ($P < 0.05$) in the presence of iAs(III) compared to no iAs(III) controls (Figure 2a and Table S13). Among the treatments with iAs(III), we observed that *arsM* gene transcript copy numbers exhibited an opposing trend to substrate content: 25% RCB > 50% RCB > 75% RCB > 100% RCB (Figure 2a). The highest number of *arsM* transcripts was detected in the most dilute medium (25% RCB + iAs(III), 1.04E+05 ± 2.05E+04 copies/OD_{600}), which was approximately 4, 5, and 12 times greater than in the treatments of 50% RCB + iAs(III) (2.63E+04 copies/OD_{600}), 75% RCB + iAs(III) (2.11E+04 copies/OD_{600}), and 100% RCB + iAs(III) (8.90E+03 copies/OD_{600}), respectively (Figure 2a). This result is deemed robust because, in the absence of iAs(III), the trend follows the opposite direction, i.e., *arsM* expression is highest in the no dilution (100% RCB) condition (Figure 2a). We attribute the latter trend to imperfect normalization of *arsM* expression (we presume that the expression of *arsM* gene in all dilutions should be the same without iAs(III)) and biases stemming from the differences in expression in cells growing in substrate-replete *vs*. substrate-depleted conditions. However, these biases only strengthen the findings reported in the presence of iAs(III) because they would tend to decrease the expression of *arsM* in the higher dilution conditions, while the finding reports the highest transcript number in those conditions.

Furthermore, correlation analysis suggested a significant ($P < 0.05$) positive correlation between *arsM* gene transcripts and the concentrations of aqueous MMAs(III) (no-oxidation) (Figure 2b) and aqueous MMAs(V) (post-oxidation) (Figure 2c). Similarly, the correlation between *arsM* gene transcript number and MMAs(III) content was also

supported when using the second experimental method for absolute transcript quantification, which relies on using the same amount total RNA for reverse transcripts regardless of biomass amount used for the extraction (Figure S16 and Table S14).

Impact of strain EML on *E. coli* growth rate. Next, we sought to probe the direct impact of As methylation by strain EML on other microorganisms. Confirmation of As(III)-resistance in *E. coli*, MMAs(III)-sensitivity in WT *E. coli*, and MMAs(III)-resistance in *E. coli* expressing *arsP* (hereafter, ArsP *E. coli*) is provided in Figures S1-S3, Texts SR1-SR2, and Tables S15-17 and optimization of the co-culture ratio for incubations in Figures S4-S6, Text SR3, and Tables S18-S20. WT or ArsP *E. coli* in anaerobic co-culture with strain EML exhibited similar patterns of growth, with rapid growth within 10 hours of incubation, followed by a decline in copy number (Figure 3a and Table S21). The growth of ArsP *E. coli* was significantly higher ($P < 0.01$) than that of WT *E. coli* (Figure 3a), while the growth of strain EML did not significantly differ between the two co-culture treatments (Figure 3b). Thus, the growth difference between WT *E. coli* and ArsP *E. coli* in anaerobic co-culture with strain EML cannot be explained by growth-rate differences in strain EML during the co-culture period. We propose that the growth rate difference is attributable to the production of toxic MMAs(III) that inhibits the growth of WT *E. coli*, but negligibly affects that of ArsP *E. coli* (Figures S1 and S2). In addition, the growth of strain EML alone was greater than that of strain EML in co-culture with either *E. coli* strain (Figure 3b), and it could also be reasonably attributed to the competition for growth substrate in co-culture.

MMAs(III) production and *arsM* gene transcription during co-culture. According to the microbial warfare hypothesis, we would expect higher MMAs(III) production by strain EML when co-culturing with ArsP *E. coli* than WT *E. coli*, in order to thwart competition for nutrients with the faster growing strain. Indeed, MMAs(III) was found to

be the dominant methylated As species and it increased gradually along with the decrease of iAs(III) during the co-culture period (Figure S17 and Table S22). After normalization to biomass (16S rRNA gene copy number) (Figure 4a and Table S23), we observed that MMAs(III) concentrations increased in the following order: strain EML < strain EML + WT *E. coli* < strain EML + ArsP *E. coli* (Figure 4a).

RT-qPCR was further used to investigate how substrate competition would impact the transcription of the *arsM* gene in anaerobic co-culture systems. The relative abundance of transcribed *arsM* gene in strain EML was measured during the exponential growth phase (4 h and 6 h) in anoxic co-culture systems (Figure 4b). It is clear that the transcription of *arsM* gene in strain EML (normalized to its 16S rRNA gene copy number) was significantly ($P < 0.05$) higher in co-culture with ArsP *E. coli* than WT *E. coli* at 4 h, at mid-exponential phase (Figure 4b and Table S23). This difference in expression is consistent with more MMAs(III) produced in the strain EML and ArsP *E. coli* co-culture than that in the strain EML and WT *E. coli* system (Figure 4a). Additionally, strain EML alone also produces less MMAs(III) than either *E. coli* co-culture system but its *arsM* expression does not significantly differ from that of the co-culture including WT *E. coli* (Figure 4b).

DISCUSSION

In this study, we provide evidence that trivalent monomethylated As, MMAs(III) is produced as the dominant methylated As species by the recently isolated anaerobic As-methylating bacterium, *Paraclostridium bifermentans* strain EML, during growth in the presence of iAs(III) (Figure 1). The concentration of MMAs(III) increases during the exponential growth phase and remains stable in the stationary phase (Figure 1). This strongly suggests that anaerobic As methylation resulted from the activity of a

functional ArsM from strain EML, rather than by the fortuitous methylation of As owing to the release of methyltransferases upon cell lysis as evidenced for methanogens in a previous study.[25] An *arsM*-containing *ars* operon (*arsM-acr3-MPPE-arsR*1) was identified in strain EML (Figure S18), supporting this interpretation.

A major question remains: what is the ecological function of generating a product (MMAs(III)) that is more toxic than the substrate (iAs(III))? At first glance, this would appear to be deleterious to the microorganism. However, this process could result in a beneficial outcome if two conditions are fulfilled. The first condition is that trivalent methylated As compounds serve as antibiotics to inhibit other potentially competing microorganisms.[19] Because MMAs(III) and DMAs(III) are thermodynamically stable under anoxic conditions, they persist sufficiently long to be effective as antibiotics for anaerobes. The second condition is that MMAs(III), which is produced intracellularly, is exported to the extracellular space, precluding self-toxicity. If the rate of efflux of MMAs(III) is greater than that of iAs(III), As methylation would represent a net detoxification process.

The conditions propitious for anaerobic As methylation remain elusive. A comparative study of As methylation across aerobic and anaerobic microorganisms revealed that, despite encoding a functional ArsM, anaerobes did not necessarily methylate iAs(III).[25] This observation was partially attributed to the efficient efflux of iAs(III) in anaerobes (but not in aerobes), precluding sufficient accumulation of iAs(III) intracellularly for methylation to occur.[34] Knocking out the *acr3* gene, encoding the iAs(III) efflux pump in the anaerobe *Clostridium pasteurianum*, resulted in an obvious increase in intracellular iAs(III) but, overall, the As methylation efficiency improved little.[25] A recent paper showed an increase in As methylation (under oxic conditions) upon knocking out the iAs(III) transporter gene *arsB* in cells of *E. coli* expressing

arsM.[34] Nonetheless, while iAs(III) influx is an important control on As methylation, we surmised that, in addition to intracellular iAs(III), other factors may control anaerobic As methylation. If the microbial warfare hypothesis for anaerobic As methylation holds, anaerobic As methylation might be triggered by an environmental signal suggesting obstacles to optimal growth (e.g., limited resources) or by specific metabolites produced by other microbial community members signaling their presence as potential competitors.[21, 25]

Here, the first aim was to investigate the microbial warfare hypothesis for anaerobic As methylation by generating growth substrate limitations. This hypothesis was probed by growing strain EML in dilutions of RCB medium and measuring the extent of As methylation. We found opposing trends between growth substrate content and concentration of aqueous MMAs(III) (Figure 1) or *arsM* gene transcript numbers (Figure 2). Strain EML grown at lower growth substrate conditions produced higher (protein-normalized) concentrations of MMAs(III) (Figure 1), and exhibited higher (protein-normalized) expression of the *arsM* gene (Figures 2 and S14), suggesting that the cells responded to growth substrate limitation by increasing As methylation. Taken together, these results demonstrate an important role for growth substrate availability in regulating anaerobic As methylation by strain EML. In addition, we observed the accumulation of intracellular As(III) preferentially in the low-growth substrate conditions, confirming the close relationship between intracellular iAs(III) and high As methylation potential, as previously evidenced.[25]

Next, direct evidence of microbial inhibition by microbial MMAs(III) production was sought by the anaerobic co-culture of strain EML with either MMAs(III)-sensitive WT *E. coli* or MMAs(III)-resistant *E. coli* (i.e., ArsP *E. coli*) in RCB medium (Figures 3 and 4). We found a two-way interaction between strain EML and *E. coli*: on the one hand,

MMAs(III) produced by strain EML inhibits the growth of WT *E. coli* to a greater extent than that of ArsP *E.coli* (Figure 3), supporting the microbial warfare hypothesis. On the other hand, we interpret less growth by WT *E. coli* to be the result of lower substrate depletion as compared to ArsP *E. coli* and, in turn, lower expression of *arsM* in strain EML (Figure 4). However, it is also conceivable that other factors (e.g., signaling) cause lower *arsM* expression. Therefore, it is reasonable to conclude that MMAs(III) production inhibits microorganisms unprepared to detoxify it and that strain EML responds to substrate limitation by increasing *arsM* expression and, thus MMAs(III) production. While more MMAs(III) is produced by strain EML co-cultured with ArsP *E. coli*, the growth of strain EML is comparable in the presence of either *E. coli* strain (Figure 3b). A proposed explanation for this observation is that, while MMAs(III) production is a response to substrate limitation, it does not necessarily thwart the competitor (i.e., the prey) sufficiently to impact resource use, and thus growth. Indeed, the impact of resource competition is evident from comparing the growth of strain EML with or without *E. coli* (Figure 3b).

Efflux of MMAs(III) is required for effective delivery of antibiotics to other microorganisms and, to avoid self-toxicity (as stated in condition 2 above). Specific/nonspecific MMAs(III)-pump genes *arsP*[30] and *arsK*[35] are known and the co-evolution of *arsM* and *arsP* was previously evidenced, suggesting a strategy of MMAs(III) efflux by MMAs(III)-producing and MMAs(III)-resistant microorganisms.[18-20] The identification of two chromosomally encoded *arsP* genes in strain EML indicates that is may be capable of effluxing MMAs(III) to the extracellular space (Figure S18) and variable *arsP* expression across conditions (not measured) may account for the similarity in the extent of *arsM* expression by strain EML growth with *E. coli* WT and strain EML alone.

In this study, we provide direct evidence of the role of growth substrate competition in anaerobic As methylation by strain EML, supporting the proposed microbial warfare strategy. Furthermore, we evidence a feedback-loop, by which a bacterium resistant to MMAs(III) enhances its production, presumably through enhanced *arsM* expression as a result of substrate limitation. Therefore, the work uncovers complex interaction between an anaerobic As methylator and potential competitors. Further work is clearly needed, first to uncover the mechanism of regulation of *arsM* gene expression by the growth substrate concentration and second to elucidate other factors that may control anaerobic As methylation. Substantial understanding of the controls on anaerobic As methylation is required for the development of strategies to limit As methylation in rice paddy soils.

ASSOCIATED CONTENT

Supporting Information

Texts: Methods: MMAs(III) abiotic controls; *arsM* gene primer design; construction of *arsM* plasmid standards, and anaerobic co-culture systems. Results: confirmation of As(III)-resistance in *E. coli*, MMAs(III)-sensitivity or resistance by *E. coli* strains, and optimization of the co-culture ratios; Discussion.

Figures: As(III)-resistance and MMAs(III)-resistance/sensitivity in E. coli strains; optimization of co-culture ratio; growth curves (OD_{600} and protein); aqueous and intracellular As speciation; plasmid *ars* operon; MMAs(III) chemical stability; reference genes selected by qBase plus software; *arsM* gene transcription; chromosomally encoded *arsP* gene; the unknown peak (post-oxidation); As mass balance.

Tables: Experimental conditions; reference gene primers; raw data for all experiments.

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Notes

The authors declare no competing financial interest.

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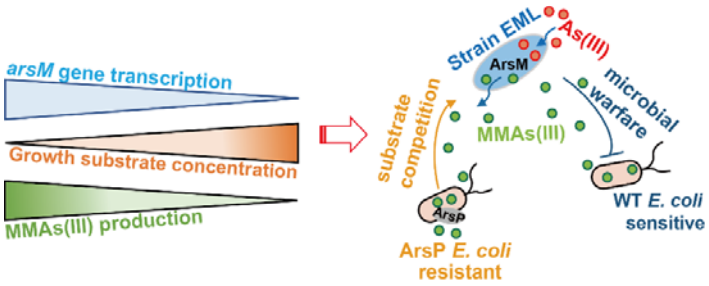
FIGURE CAPTIONS

Figure 1. Time-dependent concentrations of protein-normalized aqueous As species in anaerobic RCB dilutions (100%, 75%, 50%, or 25% RCB) inoculated with *Paraclostridium bifermentans* strain EML and 25 μM iAs(III). Individual values for each biological replicate can be found in Supporting Information Table S7. (a) MMAs(III) (no oxidation), (b) MMAs(V) (post-oxidation) and (c) DMAs(V) (post-oxidation).

Figure 2. (a) Transcripts of *arsM* gene of *Paraclostridium bifermentans* strain EML in anaerobic RCB dilutions (100%, 75%, 50%, or 25% RCB) in the presence and absence of 25 μM iAs(III) at 8 hours of incubation. (b) and (c) Correlation analysis of *arsM* gene transcripts and concentrations of MMAs(III) (no oxidation), and MMAs(V) (post-oxidation) at 8 hours of incubation. Different letters showed significant difference at $P < 0.05$. Individual values for each biological replicate are shown in Supporting Information Table S13.

Figure 3. (a) Growth curves (16S rRNA gene copy number) of *Escherichia coli* K-12 wild-type strain MG1655 (WT *E. coli*) and engineered WT *E. coli* harboring a MMAs(III)-resistance gene (*arsP*) (ArsP *E. coli*) in anaerobic co-culture with *Paraclostridium bifermentans* strain EML in anoxic RCB with 25 μM iAs(III). (b) Growth curves (16S rRNA gene copy number) of *Paraclostridium bifermentans* strain EML in anerobic co-culture systems as described above. Two-star symbols represent statistical significance at $P < 0.01$. Individual values for each biological replicate are shown in Supporting Information Table S21.

Figure 4. (a) Time-dependent concentration of 16S rRNA gene copies-normalized aqueous MMAs(III) in anaerobic co-culture *Paraclostridium bifermentans* strain EML with either WT *E. coli* or ArsP *E. coli* in anoxic RCB with 25 μM iAs(III). (b) Transcripts of 16S rRNA gene copies-normalized *arsM* gene of strain EML in anaerobic co-culture systems as described above at 0, 4, and 6 hours of incubation. Different letters indicate significant difference at $P < 0.05$. Individual values for each biological replicate are shown in Supporting Information Table S23.



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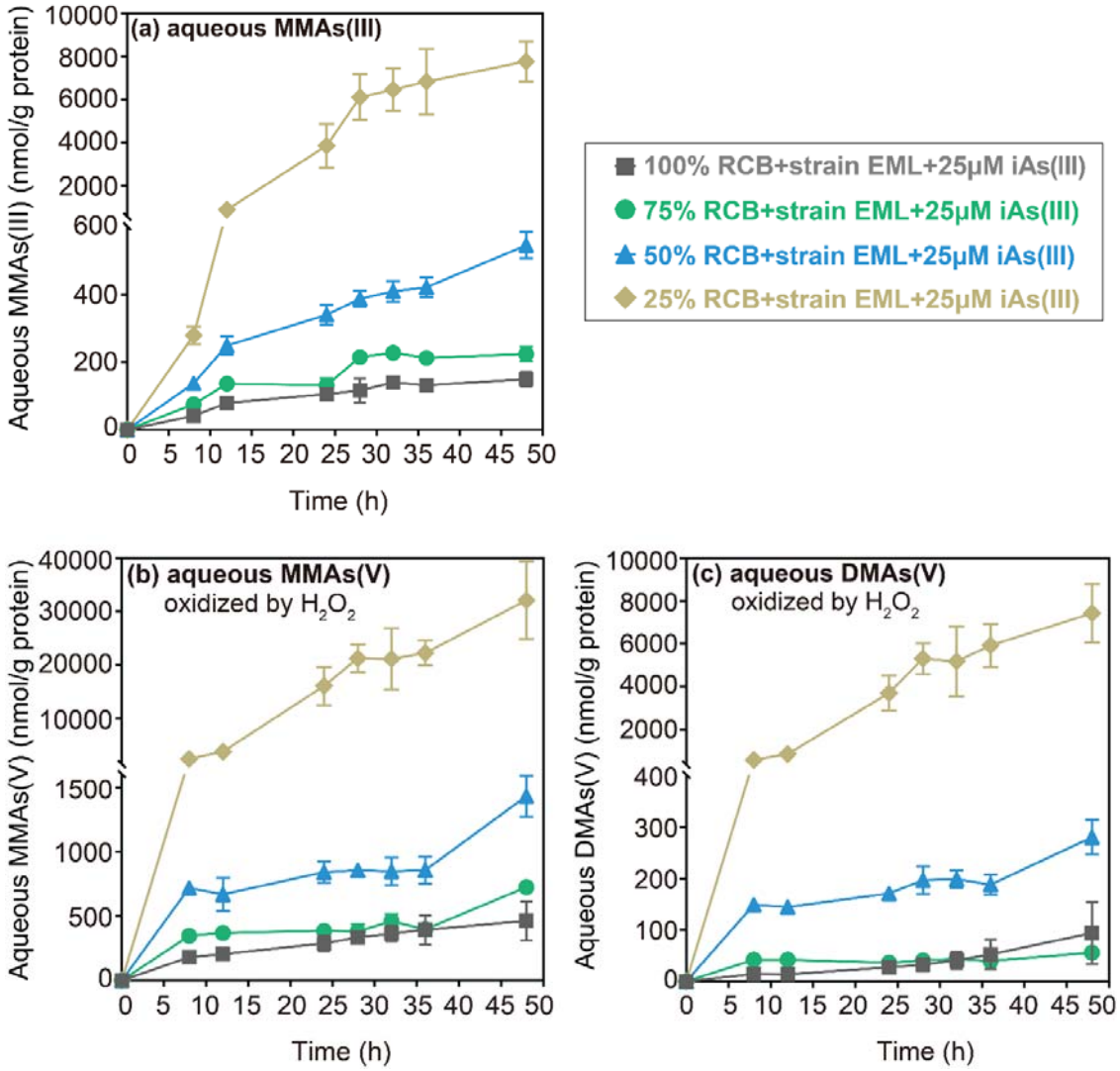

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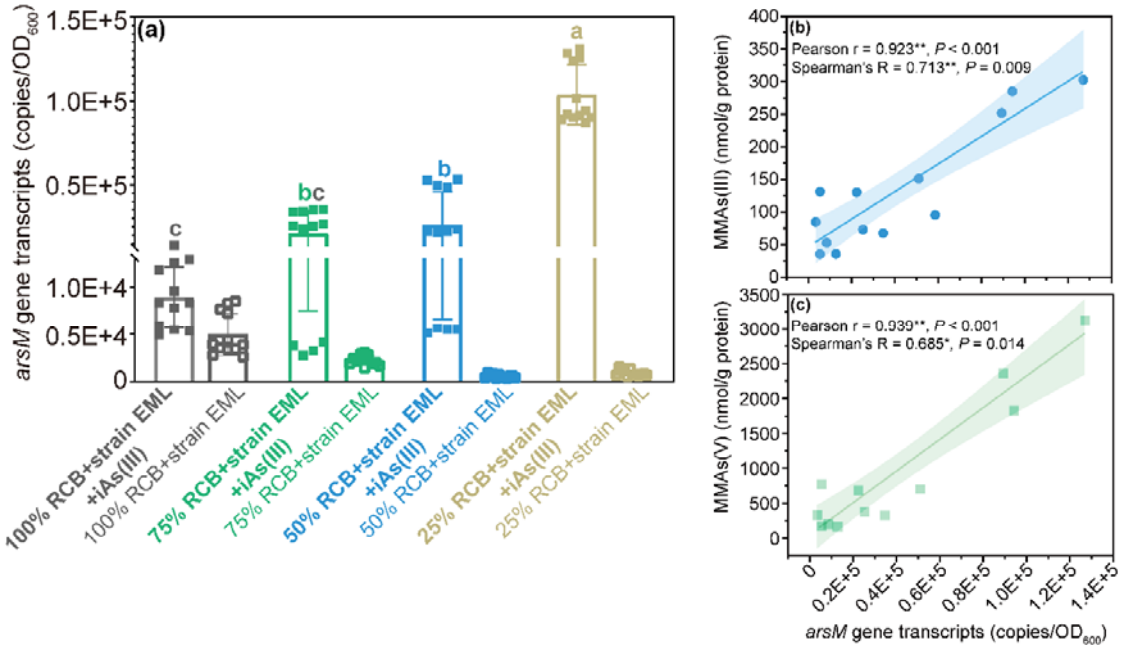

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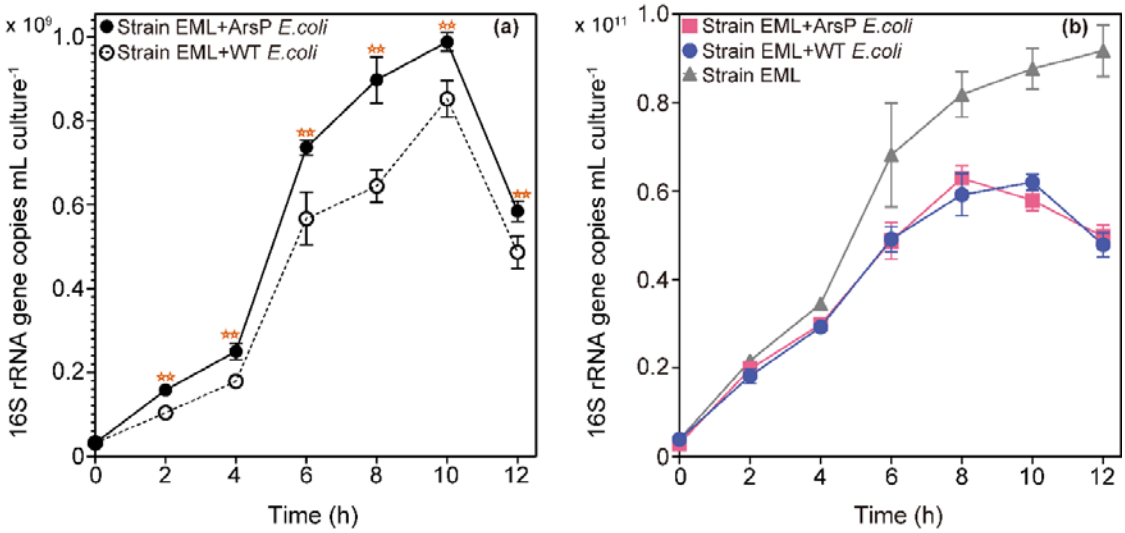

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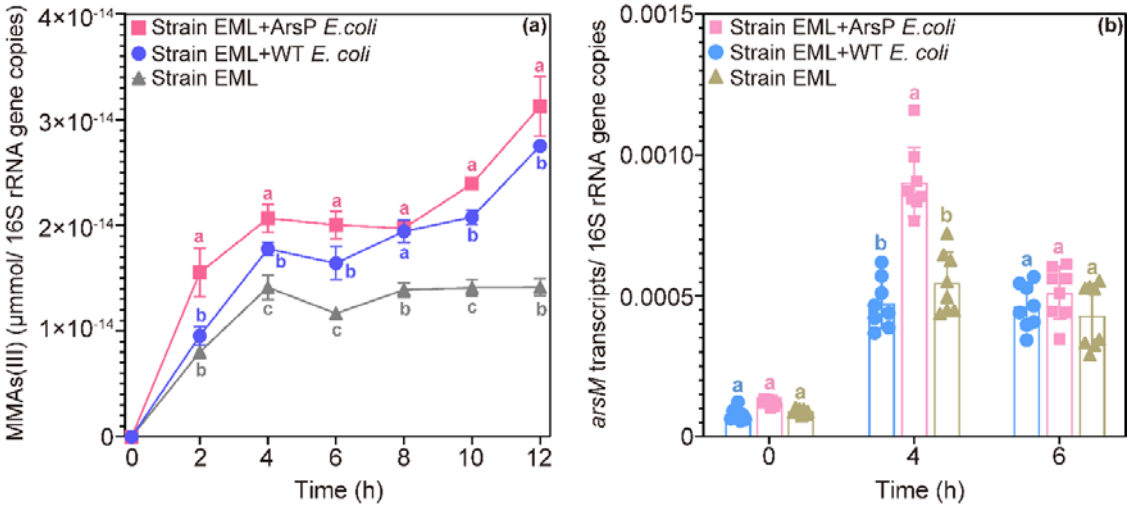

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