The Effect of Growth Substrate Availability on Anaerobic Arsenic Methylation by *Paraclostridium bifermentans* strain EML

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ABSTRACT

Microbial arsenic (As) methylation is established as a detoxification process under aerobic conditions (converting arsenite) to monomethylarsonic acid (MMAs(V))) 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 recently isolated anaerobic As methylator, *Paraclostridium bifermentans* strain EML to gain insights into this process. Strain EML was inoculated into a series of media that consisted of systematic dilutions of Reinforced Clostridial Broth (RCB) supplied with 25 μM arsenite to assess the impact of growth substrate availability on As methylation. Growth curves evidenced the sensitivity of strain EML to arsenite, and As speciation analysis revealed the production of MMAs(III). Concentrations of MMAs(III) were found to be positively corelated with the RCB medium dilution, suggesting that growth substrate availability plays an important role in the regulation of MMAs(III) production. Further, reverse-transcription quantitative PCR evidenced *arsM* gene transcription increased with RCB dilution. Thus, growth substrate limitation is interpreted to enhance *arsM* gene expression and the associated anaerobic As methylation. We propose that growth substrate competition between microorganisms may also lead to an increase in As methylation, providing support for a microbial warfare function for this process.

Key words: anaerobic As methylation, MMAs(III), *arsM* gene transcript, microbial warfare

SYNOPSIS: Understanding the controls on anaerobic microbial arsenic methylation is relevant for rice paddy soils and the accumulation of arsenic in rice grains.

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 compunds.[1-3, 5, 6] In recent years, microbial transformations of As in paddy soils have also 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] The accumulation of DMAs(V) has been shown to correlate with rice straight-head disease.[11, 12]

Arsenic methylation is a microbially-mediated process involving the transformation of trivalent inorganic As (arsenite, As(III)) into mono-, di-, and trimethylated As compounds and is catalyzed by *S*-adenosyl-methionine methyltransferase (ArsM in prokaryotes).[13-15] Under flooded conditions, the paddy soil is anoxic, favoring the activity of anaerobic microbial As methylators, and, in turn, leading to greater accumulation of methylated As in rice grains as compared to dry cultivation conditions.[16, 17] Generally, As methylation occurring under oxic conditions is proposed as an As(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 condition.[17, 18]

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.[19, 20] This finding suggests that the original function of MMAs(III) could have been to serve as a primitive antibiotic.[21, 22] At present, there is only one confirmed and available anaerobic As-methylating microorganism, *Paraclostridium bifermentans* strain EML, a fermenter isolated from a paddy soil in Vietnam.[23] However, controls over its methylating activity remains unknown.

In microbial communities, competitive phenotypes (including production of antibiotics) can arise as a consequence of limited resources.[24] Soils represent an ecosystem in which a large number of microorganisms compete for scarce resources, thus competition is widespread.[25] We hypothesized that competition for resources may boost As methylation by *P. bifermentans* strain EML, conferring it an advantage over its competitors. In this study, we aimed to investigate the effect of growth substrate limitation on As methylation to test the microbial warfare hypothesis. From an ecological point of view, it is indeed reasonable to expect enhanced As methylation under substrate-limiting conditions, as an 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.

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[26] by using a multi-omics approach targeting the MAGs harboring an expressed *arsM* gene.[23] 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 As(III) as sodium arsenite (or into the equivalent no-As(III) control) in triplicate using sterile N_2-flushed syringes and needles. The inoculum represented 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 SI.

As 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 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. To sample 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, 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 in the manner 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.[23] Six As standards were available: MMAs(III) as methyldiiodoarsine (Santa Cruz Biotechnology Inc.), TMAs(V)O as trimethyl

144 arsine oxide (Argus Chemicals Srl., Italy), DMAs(V) as sodium dimethylarsinate
145 (ABCR, Germany), MMAs(V) as monomethylarsonic acid (Chemservice, PA,
146 USA), As(III) as sodium arsenite ($NaAsO_2$) (Sigma-Aldrich, MO, USA),and As(V)
147 as sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$) (Sigma-Aldrich, MO,
148 USA). In addition, monomethylmonothioarsonic acid (MMMTAs(V)) was
149 synthesized as previously described.[27] Total aqueous and soluble intracellular As
150 concentrations were measured using the same ICP-MS instruments in stand-
151 alone mode.[23]

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

169 was used to generate a standard curve. All samples were run in quadruplicates. A
170 NRT (no-reverse transcriptase control) and a NTC (no template control) were both
171 included as negative controls.

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

190

191 **RESULTS**

192 **As(III) inhibits strain EML growth.** Anaerobic growth of *Paraclostridium*
193 *bifermentans* strain EML was conducted with various concentrations of RCB

(100%, 75%, 50%, or 25% RCB) in the presence and absence of 25 μM As(III). The growth curves show that strain EML grew rapidly and reached the mid-exponential phase after about 8 hours in the absence of As(III) (Figure S1 and Table S3). In contrast, the extent of growth of strain EML was lower in the presence of As(III). In addition, it reached the stationary and death phases sooner than in the absence of As(III) (Figure 1), particularly in the low growth substrate media (50% and 25% RCB) (Figure S1 and Table S4), indicating that the growth of strain EML is negatively impacted by As(III). While strain EML harbors a gene encoding an As(III) efflux pump (*acr3*) (Figure S2), we hypothesize that it may not be efficient enough to pump out intracellular As(III).

MMAs(III) is produced by strain EML. To profile the dynamics of As(III) transformation during anaerobic growth of strain EML, time-dependent changes in aqueous and soluble intracellular As speciation 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) under variable growth substrate conditions (Figures 2 and S3a-d and Table S5). MMAs(III) also represented the dominant methylated aqueous As species, and concentrations of MMAs(III) increased concomitantly with the decrease in aqueous As(III) (Figure S3a-d). RCB amended with 25 μM As(III) exhibits no transformation of As(III) (data not shown), therefore, our results demonstrate that this anaerobic As-methylating microorganism produces MMAs(III).

However, the chemical stability of MMAs(III) is often limited, even under anoxic conditions. 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 evidenced its significant disappearance from solution (after 24 h) (Figures S4 and S5 and Tables S6 and S7). Surprisingly, MMAs(III) stability was seemingly negatively correlated with the medium dilution (Figure S4). 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 diluted RCB medium.

The disappearance of MMAs(III) from the solution was puzzling and we hypothesized the formation of methylated and thiolated As species (e.g., monomethyldithioarsenate, MMDTAs(V)) that are not identifiable analytically in our system. To probe this possibility, we analyzed As speciation after oxidation of trivalent As species by H_2O_2, an established strategy.[28-30] Oxidation is expected to transform MMAs(III) into MMAs(V) quantitatively. As expected, after oxidation, the concentration of MMAs(V) was greater than that of MMAs(III) measured prior to oxidation (Figure S3 and Table S5). We propose that compounds such as MMDTAs(V) are formed chemically through reaction of reduced sulfur compounds with MMAs(III). These chemical species are retained by the HPLC column but are eluted after oxidation.[31] Thus, MMAs(V) in the oxidized sample corresponds to the sum of MMAs(III) and methylated and thiolated As species. Direct evidence of the formation of unknown, column-retained species is provided by the reaction of MMAs(III) with sulfide. Indeed, MMAs(III) disappears from solution (Figure S6 and Table S8), and MMMTAs(V) is formed. On the other hand, in the oxidized samples, all initial MMAs(III) is recovered in the form of MMAs(V) (Figure S6).

In the oxidized samples, a low concentration of DMAs(V) (Figure S3e-f) and an unknown As peak (Figure S7) were also detected. Similarly to above, we

propose that a thiolated and/or methylated species may be generated and retained in the anion-exchange column. However, upon reaction with H_2O_2, it is partially oxidized to pentavalent methylated As species (DMAs(V)) and readily eluted and separated. The species corresponding to the abovementioned unknown peak has not been identified. Arsenic mass balance was evaluated either by calculating the sum of all As species detected or by measuring total As (using ICP-MS) (Figure S8 and Table S9). The overall mass balance for As in our study is around 74%-118% (no oxidation) and 89%-150% (oxidation), suggesting that chemical oxidation of the sample recovers more of the As chemical species, albeit in altered form.

Impact of variable substrate concentrations. Strain EML exhibited variable growth rates for varying RCB dilutions, 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 2 and Table S10) reveals a trend in normalized MMAs(III) or MMAs(V)/DMAs(V) concentration as a function of RCB dilution (Figure 2a). Indeed, the normalized concentration decreased in the following order: 25% RCB > 50% RCB > 75% RCB > 100% RCB (Figure 2a). 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 2a). Similar patterns were also observed in the oxidized samples, with the highest protein-normalized concentrations of MMAs(V) and DMAs(V) generated in the highest RCB dilution (25% RCB) (Figure 2b and 2c). 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 S9 and Tables S11 and S12).

***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 S10). We presume that the considerable variation in growth rate due to the 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 As(III) compared to no As(III) controls (Figure 3a and Table S13). Amongst the treatments with As(III), we observed that *arsM* gene copy numbers exhibited an opposing trend to substrate content: 25% RCB > 50% RCB > 75% RCB > 100% RCB (Figure 3a). The highest number of *arsM* transcripts was detected in the lowest nutrient media (25% RCB + As(III), 1.04E+05 ± 2.05E+04 copies/OD_{600}), which was approximately 4, 5, and 12 times higher than in the

treatments of 50% RCB + As(III) (2.63E+04 copies/OD_{600}), 75% RCB + As(III) (2.11E+04 copies/OD_{600}), and 100% RCB + As(III) (8.90E+03 copies/OD_{600}), respectively (Figure 3a). This result is deemed robust because, in the absence of As(III), the trend follows the opposite direction, i.e., *arsM* expression is highest in the no dilution (100% RCB) condition (Figure 3a). We attribute this trend to imperfect normalization of *arsM* expression (we presume that the expression of *arsM* in all dilutions should be the same without As) 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 As(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 3b) and aqueous MMAs(V) (post oxidation) (Figure 3c). 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 S11 and Table S14). In summary, our findings indicate that growth substrate availability impacts the expression of *arsM* in the anaerobic As-methylating strain EML, in turn, affecting the concentration of MMAs(III) produced.

DISCUSSION

In this study, we provide evidence that trivalent monomethylated As, MMAs(III) is

319 produced as the dominant methylated As species by the recently isolated
320 anaerobic As-methylating bacterium, *Paraclostridium bifermentans* strain EML,
321 during anaerobic growth in the presence of As(III) (Figures 1 and 2). The
322 concentration of MMAs(III) increases during the exponential growth phase and
323 remains stable in the stationary phase (Figures 1 and 2). This strongly suggests
324 that anaerobic As methylation resulted from the activity of a functional ArsM from
325 strain EML, rather than by the fortuitous methylation of As owing to the release of
326 methyltransferases upon cell lysis as evidenced for methanogens in a previous
327 study.[32] An *arsM*-containing *ars* operon (*arsM-acr3-MPPE-arsR*1) was identified in
328 strain EML (Figure S2), supporting this interpretation. A question remains: why
329 would strain EML generate a product (MMAs(III)) that is more toxic than As(III)? At
330 first glance, this would appear to be deleterious to the microorganism. However,
331 this process could result in a beneficial outcome if two conditions are fulfilled. The
332 first condition is that trivalent methylated As compounds serve as antibiotics to
333 inhibit other potentially competing microorganisms.[21] Because MMAs(III) and
334 DMAs(III) are thermodynamically stable under anoxic conditions, they persist
335 sufficiently long to be effective as antibiotics for anaerobes. The second condition
336 is that MMAs(III), which is produced intracellularly, is exported to the extracellular
337 space, precluding self-toxicity. If the rate of efflux of MMAs(III) is greater than that
338 of As(III), As methylation would represent a net detoxification process.

339 The conditions propitious for anaerobic As methylation remain elusive. A
340 comparative study of As methylation across aerobic and anaerobic
341 microorganisms revealed that, despite encoding a functional ArsM, anaerobes did
342 not necessarily methylate As(III).[32] This observation was partially attributed to the
343 efficient efflux of As(III) in the anaerobes and not in the aerobes, precluding

sufficient accumulation of As(III) intracellularly for methylation to occur. Knocking out the *acr*3 gene, encoding the As(III) efflux pump in the anaerobe *Clostridium pasteurianum*, resulted in an obvious increase in intracellular As(III) but, overall, the As methylation efficiency improved little.[32] Therefore, we surmised that in addition to intracellular As(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.[23, 32]

Here, we aimed at simulating growth substrate limitation by growing the anaerobic As methylator, strain EML, in dilutions of RCB medium and probed the extent of As methylation. We found opposing trends between growth substrate content and concentration of aqueous MMAs(III) (Figures 1 and 2) or *arsM* gene transcript numbers (Figure 3). Strain EML grown at lower growth substrate conditions produced higher (protein-normalized) concentrations of MMAs(III) (Figure 2), and exhibited higher (protein-normalized) expression of the *arsM* gene (Figures 3 and S12), 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 As(III) and high As methylation potential, as previously evidenced.[32] The findings are consistent with a microbial warfare strategy by which, under growth substrate-limiting conditions, production

of an antibiotic would provide a competitive advantage.

However, 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* [33] and *arsK* [34] 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[20-22, 33, 35] The identification of two chromosomally encoded *arsP* genes in strain EML indicates that it may be capable of effluxing MMAs(III) to the extracellular space (Figure S12).

In this study, we provide indirect evidence of the role of growth substrate limitation in anaerobic As methylation. Lower growth substrate levels induced the upregulation of *arsM* gene transcription, leading to a higher production of MMAs(III) (and derivatives resulting from chemical reactions). This finding was interpreted as a strategy to inhibit other microorganisms with an arsenical antibiotic. Presumably, growth substrate competition would lead to substrate limitation that, in turn, results in the production of MMAs(III) with its attendant toxic effect on competitors.

However, direct evidence of microbial warfare under anoxic conditions remains to be shown. Besides growth substrate availability, other factors such as metabolites produced by other microbial community might also play a critical role in controlling anaerobic As methylation. Additionally, the mechanism of regulation of *arsM* gene expression by the growth substrate concentration remains a knowledge gap. Further work is required to elucidate other factors controlling anaerobic As methylation and their mechanism to help develop potential strategies to limit As methylation in rice paddy soils.

ASSOCIATED CONTENT

Supporting Information

Materials and methods: MMAs(III)-associated abiotic controls; *arsM* gene primer design; construction of *arsM* plasmid standards.

Figures: Growth curves (OD_{600}); aqueous As species; stability of MMAs(III); As mass balance; reference genes selected by qBase plus; *arsM* gene transcription. plasmid *ars* operon, non-normalized aqueous As species with and without oxidation; the stability of MMAs(III) in fresh/spent RCB; reaction of MMAs(III) with sulfide; the unknown peak (post-oxidation); As mass balance; normalized intracellular As; reference genes selected by qBase plus software; transcriptions of *arsM* genes and correlation analysis; chromosomally encoded *arsP* gene.

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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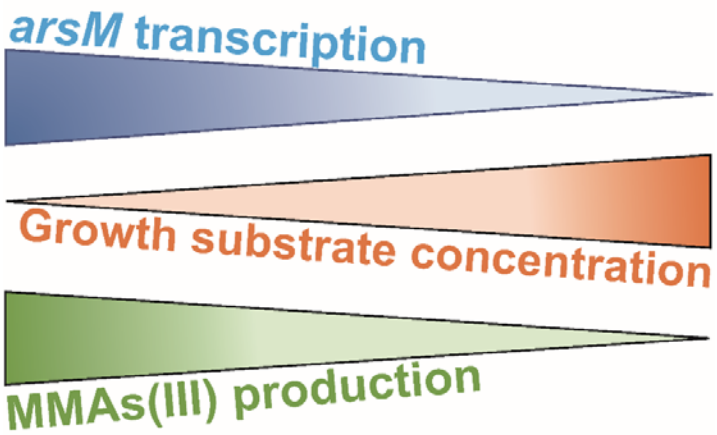
FIGURE CAPTIONS

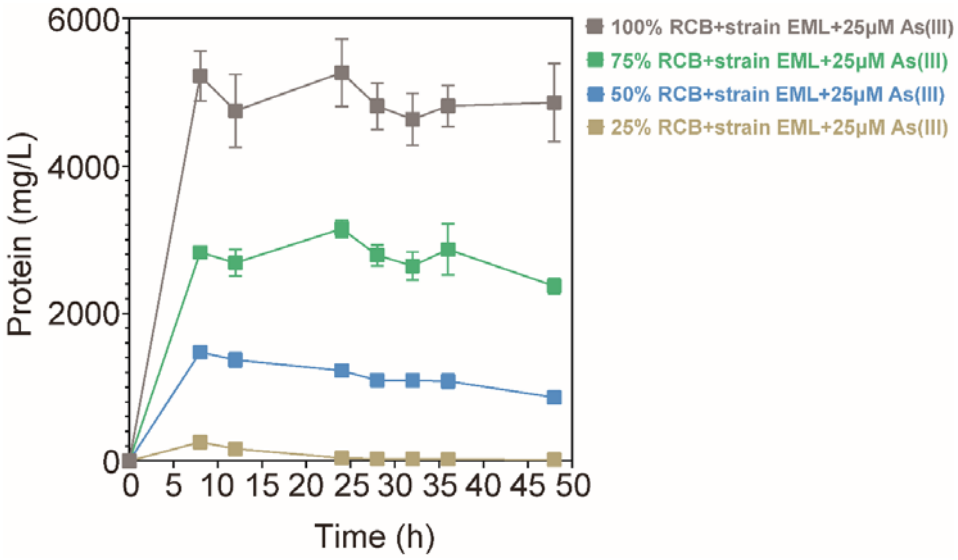
Figure 1. Growth curves (total protein) of *Paraclostridium bifermentans* strain EML in anoxic RCB dilutions (100%, 75%, 50%, or 25% RCB) in the presence of 25 μM As(III). Data are shown as mean values with error bars. Individual values for each biological replicate are included in Supporting Information Table S4.

Figure 2. Time-dependent concentrations of protein-normalized aqueous As species in anoxic RCB dilutions (100%, 75%, 50%, or 25% RCB) inoculated with *Paraclostridium bifermentans* strain EML and 25 μM As(III). Individual values for each biological replicate can be found in Supporting Information Table S10. (a) MMAs(III) (no oxidation), (b) MMAs(V) (post-oxidation) and (c) DMAs(V) (post-oxidation).

Figure 3. (a) Transcripts of *arsM* gene of *Paraclostridium bifermentans* strain EML in anoxic RCB dilutions (100%, 75%, 50%, or 25% RCB) in the presence and absence of 25 μM As(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.



Graphical abstract



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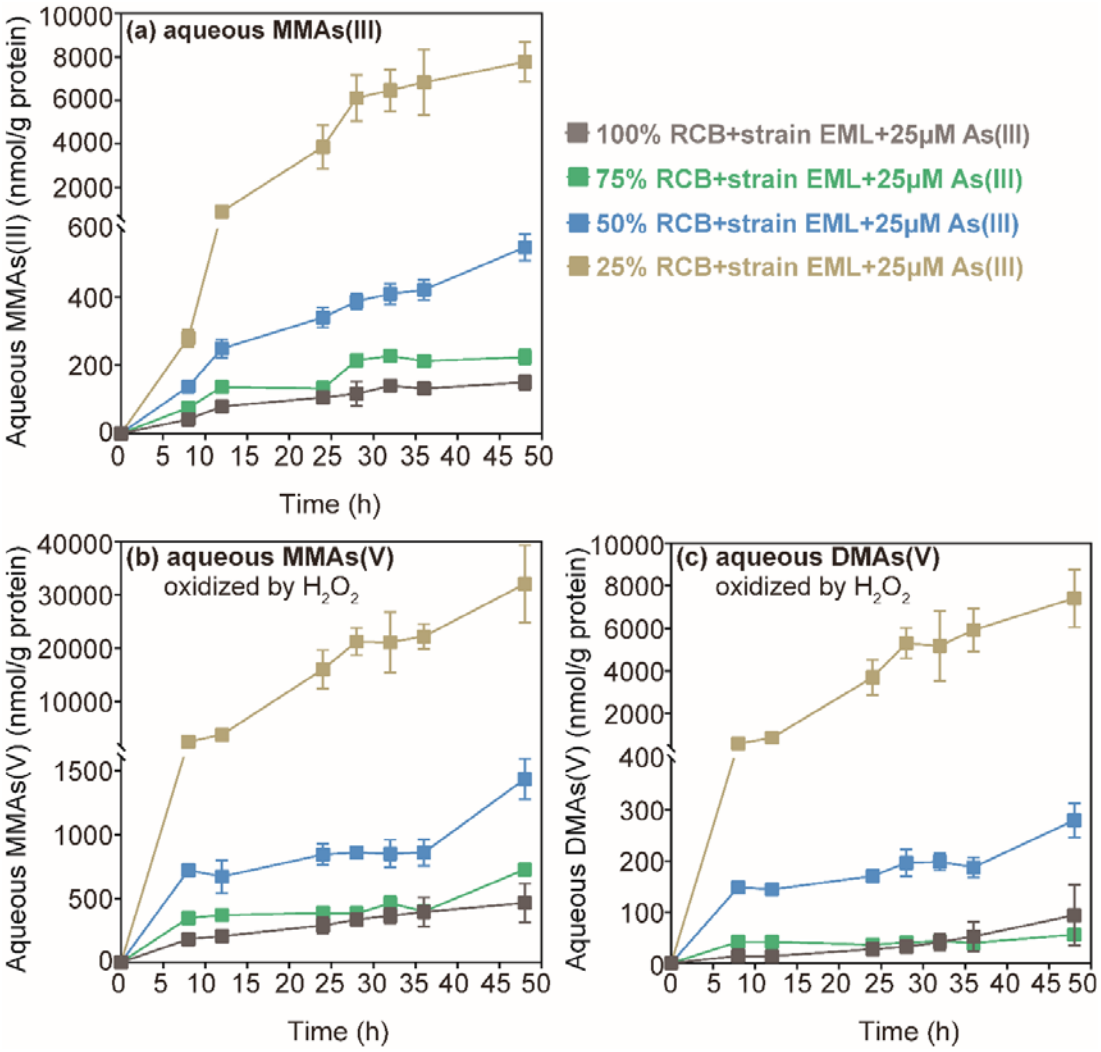

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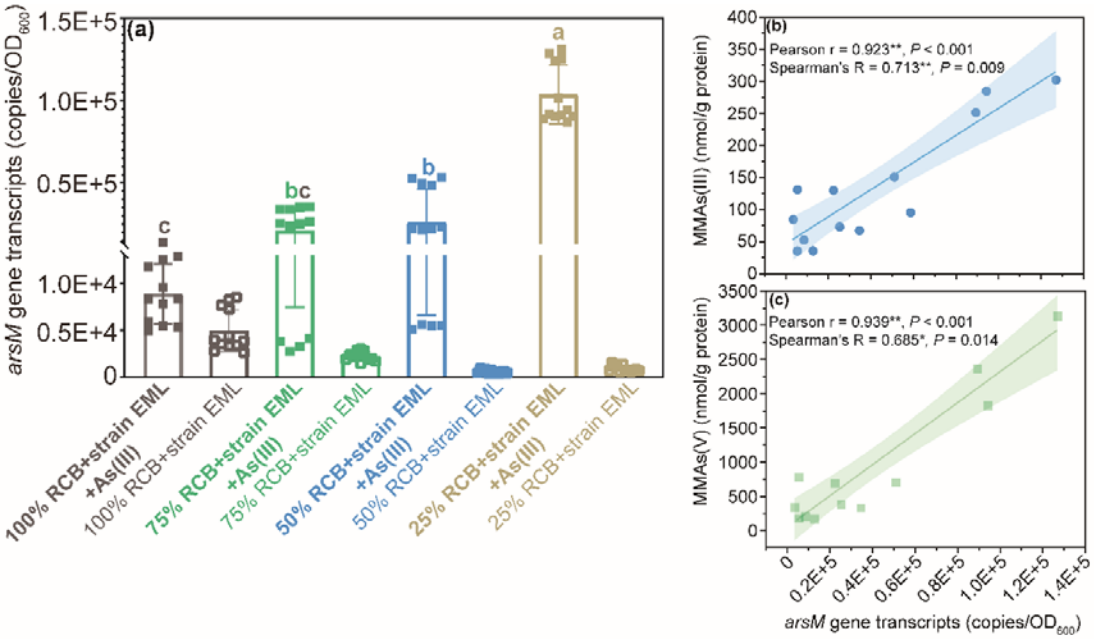

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