Conversion of Escherichia coli to Oxidize Methane for Reduction of Bovine Methane Pollution in Agriculture

A Technical Report submitted to the Department of Biomedical Engineering

Presented to the Faculty of the School of Engineering and Applied Science

University of Virginia

Charlottesville, Virginia

In Partial Fulfillment of the Requirements for the Degree

Bachelor of Science, School of Engineering

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Spring 2023

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Abstract

Livestock production accounts for 14.2% of global greenhouse gas (GHG) emissions. Specifically, dairy cattle and other ruminants contribute 4% percent and meat cattle contribute an additional 2% in the US, primarily through enteric rumination.[1] In rumination, methanogenic microbes in the rumen ferment feedstuff, producing methane. We propose an experimental approach to modify *Escherichia coli* (*E. coli*), a harmless bacteria found in the rumen, to consume methane. We encapsulated three genes encoding for the methane-oxidizing protein, particulate methane monooxygenase (pMMO), from *Methylococcus capsulatus* in plasmid vectors and transformed *E. coli* strain BL21.[2] We promote plasmid vector uptake with a standard 12-hour culture phase and an 18-hour production phase. We examine microbial growth kinetics to validate plasmid vector agreement with *E. coli* viability. Further, we determine the utilization of pMMO in a closed environment containing high methane concentrations. Results show normal growth kinetics of modified *E. coli*. Further results show no decrease in methane concentration from *E. coli* uptake in methane-rich conditions compared to a controlled environment. Future work can implement pMMO-encoding genes in chemically competent *E. coli* with different antibiotic-targeted vectors to improve vector uptake.

Keywords: enteric rumination, *Escherichia coli* (*E. coli*), particulate methane monooxygenase (pMMO)

Introduction

Impact of Methane on the Environment

Methane (CH_4) is the primary contributor to tropospheric ozone (O_3), a dangerous greenhouse gas with detrimental effects on human, animal, and environmental health. Methane reacts with sunlight, carbon monoxide (CO), non-methane volatile organic compounds (NMVOC), and nitrogen oxides (NO_x) to produce tropospheric ozone. Estimates suggest over one million pollution-related deaths every year from cardiovascular and respiratory complications are caused by ozone exposure.[3] Additionally, ozone in the troposphere is a large contributor to global warming, accounting for 30% of global warming since the pre-industrial period. Methane is 80 times more potent at global warming than carbon dioxide.[4] Through an agricultural lens, tropospheric ozone causes up to fifteen percent of annual yield losses of staple crops, such as soy, wheat, rice, and maize. Further, ozone oxidation reduces the ability of plants to complete carbon dioxide sequestration, doubling the impact of carbon dioxide on the climate.[3] Fortunately, methane has a shorter lifetime in the atmosphere (12 years) in comparison to carbon dioxide (≥100 years); therefore, any reduction in anthropogenic methane emissions would provide benefits to human, animal, and environmental health within decades.[3,5]

Methane Emissions in Bovine Production

The bovine production industry produces methane primarily through a biological process, enteric rumination. Estimates state that one cow will emit 220 pounds of methane through rumination each year.[1] Rumination is a digestive process in which microbes in the bovine rumen ferment high-fiber forage into macronutrients for nutrient absorption.[6] The rumen bacteria undergo carbohydrate fermentation, which produces hydrogen (H_2), carbon dioxide (CO_2), and formic acid as a byproduct.[7] Rumen prokaryotic archaea, or methanogens, utilize hydrogen to reduce the carbon dioxide into methane. Methanogenic behaviors in the rumen are essential for productive fermentation.[8] Low partial pressure of hydrogen is required for the production of less reduced end products and a

reduction of methanogenic behaviors results in a decreased efficiency of nutrient uptake by the bovine.[8,9]

Previous Work in Modifying E. coli for Environmental Bioremediation

Previous work engineered a non-pathogenic strain of *E. coli* to convert carbon dioxide into biomass, thus altering the metabolism of the strain from a heterotrophic to an autotrophic metabolism. The modified *E. coli* implemented carbon fixation and reduction through the implementation of the Calvin-Benson-Bassham cycle (CBB).[10] Thus, our work seeks to answer a similar question in the field of synthetic biology and our methods are motivated by previous work in implementing the CBB cycle into an *E. coli* strain. We ask whether it is possible to implement a methane-oxidizing pathway in a non-pathogenic strain of *E. coli* to reduce the presence of methane in a closed atmospheric environment (Fig. 1).

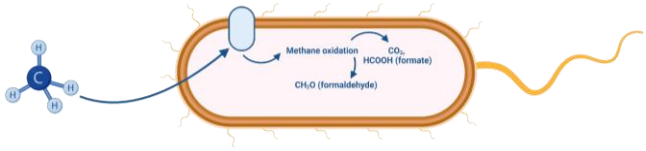

Fig. 1. Proposed Implementation of pMMO into *E. coli* strain BL21. Schematic demonstrates proposed functioning of implemented pMMO enzymatic behavior in modified *E. coli* strain BL21.

Significance of Modifying E. coli to Oxidize Methane

A global reduction of methane emissions by 45% is estimated to have significant benefits for human, animal, and environmental health. Estimates suggest that a reduction of methane emissions by 45% avoids 255,000 deaths from cardiovascular and respiratory diseases, 26 million tons of staple crop losses, and 73 billion lost work hours. Methane emissions in the agricultural sector must be reduced by 20-25% in order to support a total reduction in methane emissions of 45%, with the two other sections as fossil fuels and waste.[11] Given the large methane emissions from the bovine production sector, we propose that the implementation of a modified non-pathogenic *E. coli* strain into the bovine rumen with the ability to oxidize methane could support a reduction in methane emissions in the agricultural sector. Thus, we propose an experimental approach of genetically engineering a non-pathogenic strain of *E. coli* with plasmid vectors able to express particulate methane monooxygenase (pMMO), a methane-oxidizing enzyme common in methanogens, with the ultimate goal of implementing modified *E. coli* in bovine populations for the reduction of methane emissions in enteric rumination (Fig. 2).

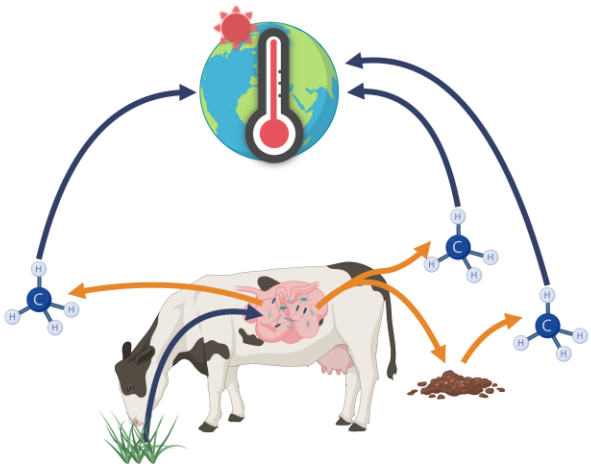

Fig. 2. Methane Emissions in Enteric Rumination. Schematic demonstrates the fermentation of high-fiber feedstuff and production of methane emissions from enteric rumination.

Project Aims and Proposed Hypothesis

Therefore, we propose multiple aims to support an experimental approach to modifying an *E. coli* strain to produce pMMO for enzymatic oxidation of methane. Aim 1 is to understand the *E. coli* genome and genes encoding for particulate methane monooxygenase (pMMO) in *Methylococcus capsulatus* through literature review. Aim 2 is to modify the selected *E. coli* BL21 strain to reduce methane into carbon dioxide with pMMO insertions from plasmid vectors. Aim 3 is to confirm the viability of modified *E. coli* through growth kinetics studies. Aim 4 is to validate methane consumption by modified *E. coli* through growth kinetics and methane utilization experiments with a Gas Chromatography (GC) machine. Through the four aims, we propose that successful implementation of pMMO-encoding genetic sequences into an *E. coli* BL21 population and growth under selective, methane-rich conditions will promote pMMO expression and enzymatic activity of pMMO, thus reducing methane concentrations in methane-rich atmospheric conditions.

Limitations and Design Constraints

The greatest limitation of the project is financial constraints. Due to the varying socioeconomic statuses of United States ranchers, the proposed modified *E. coli* must be able to undergo scale-up production at industrial scale while maintaining low costs. Thus, plasmid vectors were purchased from an external company at a low cost and effects on efficiency of bacterial transformations are expected. However, selective conditions in further experimental steps should filter successfully modified *E. coli*. One design constraint is the utilization of an *E. coli* BL21 strain, which was chosen based on availability of the

strain and literature review on viability of the strain for genetic modification.[12] Further, another design constraint is designing an experimental approach that maintains sterility and reduces risk of contamination so that the modified *E. coli* can proliferate without microbial competition. Overall, the proposed experimental approach for bacterial transformations, microbial culturing, and validation experiments accounts for all limitations and given constraints.

Methods

Bacterial Transformation

To induce the genetic transformation of *E. coli* strain BL21, an industry-standard protocol was implemented. A control subgroup was administered 0.5 µL of a paradigmatic pRSET vector with ampicillin-resistance green fluorescent protein (GFP) to verify bacterial uptake. Three discrete populations of chemically competent BL21 *E. coli* were treated with unique arrangements of plasmid vectors. The first BL21 experimental group was introduced to 20ng of pmoA-encoding DNA suspended in DI water. The second BL21 experimental group was introduced to 10ng of pmoA, pmoB, and pmoC-encoding DNA through simultaneously integrating three plasmid vectors into the group. The three populations were electroporated, with all populations undergoing a voltage of 1.8kV. The samples were subsequently flushed with SOC media and placed on a shaking incubator at 37°C for 30 minutes to promote plasmid vector uptake. The first BL21 experimental group was introduced to 20ng of pmoB and pmoC-encoding after 12 hours of incubation.

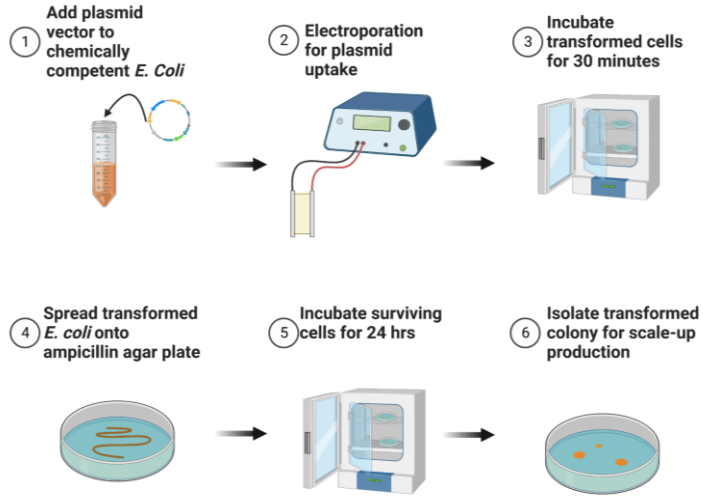

Fig. 3. Bacterial Transformation Protocol for Modification of *E. coli* strain BL21. Schematic demonstrates the industry-standard protocol for bacterial transformations for the modification of *E. coli* strain BL21.

1, 5, and 10µL of each sample were spread onto ampicillin-agar plates and incubated at 37°C over 12 hours. Single colonies were isolated from the ampicillin-agar plates and mixed with 17.5µg of ampicillin and 17mL of Luria-Bertani (LB) broth in conical tubes. Selected samples of the two experimental groups, 3-vector (simultaneous introduction of pmoA, pmoB, pmoC-encoding plasmid vectors) and 1+2-vector (introduction of pmoA-encoding plasmid vector, and secondary introduction of pmoB and pmoC-encoding plasmid vectors), were placed into a shaking incubator at 37°C for 18 hours of scale-up production. Remaining samples were placed into a 2°C refrigerator for long-term storage.

Normal Growth Kinetics with Growth-Enhancing Luria-Bertani (LB) Broth

For each modified *E. coli* group, 5 mL of the sample was added to a solution of ampicillin (75 µg) and 75 mL of LB broth and incubated in a shaking incubator at 37°C. Absorbance readings were taken every hour for a 6-hour period, using 0.9 mL per sample.

Methane-rich Atmospheric Conditions and Minimal Media Growth Kinetics

The growth kinetics of four samples were studied with minimal growth media and under consistent methane bubbling into a closed environment. Each inoculated vial consisted of 50 mL of minimal media and constant natural gas bubbling. The control population contained 5mL of 3-vectored modified *E. coli* and 5mL of 1+2-vectored modified *E. coli* under normal atmospheric conditions and in a 20°C, mixing flask. The experimental population contained 5mL of 3-vectored modified *E. coli* and 5mL of 1+2-vectored modified *E. coli* under methane-rich atmospheric conditions from natural gas bubbling into the mixing flask at 20°C. Absorbance readings were taken every hour for a 6.5-hour period, using 0.9 mL per sample.

Gas Chromatography

A closed system gas chromatography (GC) apparatus was used to quantify methane reduction from pMMO utilization within a closed environment. A combined 0.5mL sample of both experimental populations, (0.25mL of 3-vectored in minimal media, 0.25mL of 1+2-vectored in minimal media), that had previously been exposed to methane were completely isolated from external conditions and imbued with 5mL of gaseous natural gas. Air samples were extracted from the closed system and injected into the GC reader every 4 hours. Graphical peaks measuring methane concentration per unit time were integrated in order to

ascertain the total change in methane concentration. These results were normalized to a standard control sample of gaseous natural gas with no cells present.

Results

In order to implement the particulate methane monooxygenase (pMMO), we integrated three plasmid vectors into a non-pathogenic *E. coli* strain BL21 in two different methods– one integration with all three vectors in one bacterial transformation (pmoA, pmoB, pmoC) and a second integration that first incorporated the pmoA-encoding vector and then a second integration for pmoB and pmoC, each with ampicillin resistance through an industry-standard protocol. After a brief scale-up operation over 18 hours, we performed validation experiments to determine the effectiveness of the bacterial transformation and the utilization of the pMMO-encoding plasmids.

Growth Kinetics Show Normal Growth Kinetics Post-Transformation

Growth kinetics were utilized to determine cell viability after the plasmid vectors integration during bacterial transformation. Growth kinetics evaluated cell proliferation during the lag, exponential, and beginning timesteps of the stationary phase. Growth kinetics for modified *E. coli* populations demonstrated agreeable proliferation under both normal atmospheric conditions and in growth-enhancing LB broth compared to literature values (Fig. 4).[13]

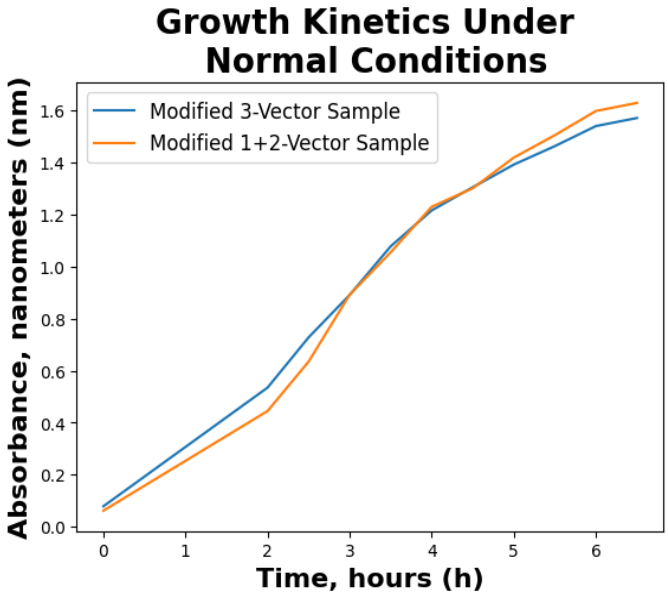

Fig. 4. Growth Kinetics under Normal Conditions. Growth kinetics were measured by obtaining optical density readings for the modified 3-vectored sample (pmoA, pmoB, pmoC in one transformation) and the modified 1+2-vectored sample (pmoA, second transformation of pmoB and pmoC.

Growth Kinetics of Modified E. coli in Methane-rich Atmospheric Conditions Show Comparable Growth Kinetics to Modified E. coli under Normal Atmospheric Conditions

Growth kinetics were examined under methane-rich atmospheric conditions and in minimal media to determine the viability of modified *E. coli* in methane-rich atmospheric conditions. Both samples contained a combined population of the modified 3-vector sample (pmoA, pmoB, pmoC in one transformation) and the 1+2-vector sample (pmoA, second transformation of pmoB and pmoC). Results indicate that plasmid integration had no appreciable effect on cell viability in comparison to normal atmospheric conditions (Fig. 5).

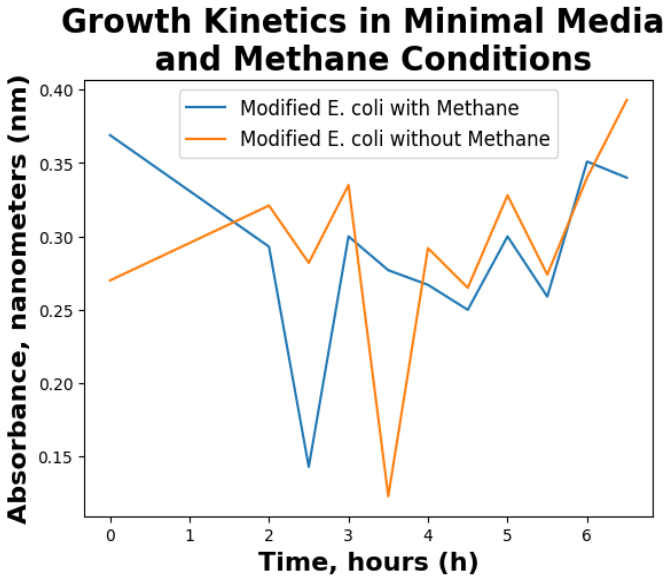

Fig. 5. Growth Kinetics in Minimal Media and Methane Conditions. Growth kinetics were measured for the modified *E. coli* (combined sample of 3-vectored and 1+2-vectored) with methane and the modified *E. coli* (combined sample of 3-vectored and 1+2-vectored) without methane by obtaining optical density readings.

Modified E. coli Did Not Utilize pMMO for Methane Oxidization in a Closed Methane-rich Atmosphere

Further results show that plasmid integration had no appreciable effect on methane concentrations in a closed methane-rich environment. Gas Chromatography techniques allow for the comparison of various gas concentrations in a closed-environment at discrete time points. Methane uptake from transformed cells is quantified by measuring the change in methane concentration in closed-environment air samples, thus indicating pMMO utilization for the oxidation of atmospheric methane. Results over 8 hours indicate no change in methane concentration over time due to pMMO utilization in the modified *E. coli* population, in which the population

contains both the modified 3-vector sample (pmoA, pmoB, pmoC in one transformation) and the 1+2-vector sample (pmoA, second transformation of pmoB and pmoC). Any decrease in methane in the *E. coli* samples is due to normal gas loss from container leakage (Fig. 6).

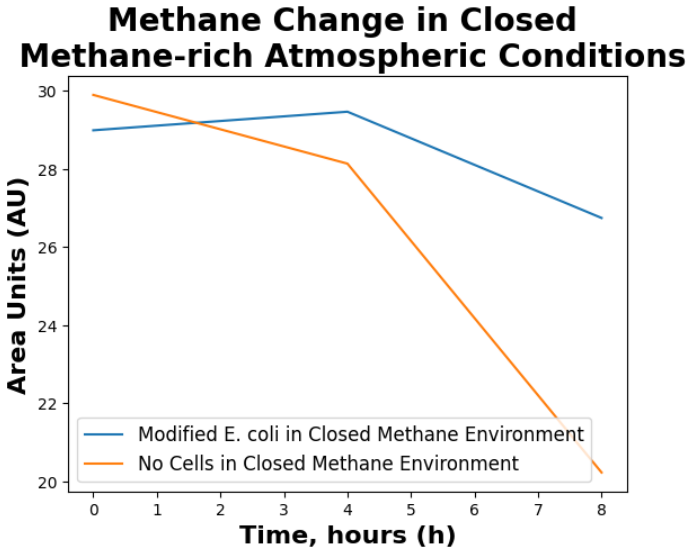

Figure 6. Methane Change in Closed Methane-rich Atmospheric Conditions. Methane concentrations are measured with a Gas Chromatography machine (GC Machine) for a combined sample of modified *E. coli* (3-vectored, 1+2-vectored) in the closed methane environment and no cells in the closed methane environment.

Discussion

Results indicate that the modified *E. coli* populations after plasmid transfection of pMMO genetic subunits maintained normal growth compared to literature values. Further, the modified populations exhibited comparable proliferation under methane-rich atmospheric conditions to modified populations under normal atmospheric conditions. There was no significant reduction in methane in closed environments by the genetically modulated groups and thus, pMMO expression and utilization cannot be verified.

Limitations

Modification of the *E. coli* with plasmid vectors did not have any effect on the viability of the cells; however, there was no appreciable change in atmospheric methane concentration from pMMO utilization. Multiple limitations were present in the experimental design. Primarily, financial constraints limited plasmid vector options from the provider, and as such, three separate vectors all contained an encoding sequence for ampicillin resistance. Thus, there were limitations on the effectiveness of the bacterial transformation due to the inability to check for each plasmid independently. Further, the expression of pMMO requires iron present in the environment. We were unable to obtain a soluble iron solute and relied on the minimal presence of iron already in the LB broth. Therefore, arguably, the environmental lack of iron could have limited the enzymatic activity of pMMO.

Future Directions

Further work could implement three vectors with three distinct antibiotic resistances to increase the efficiency of the bacterial transformation. Once validation testing confirms the successful transformations, refined experimental methods from previous work could validate protein expression within a closed methane environment containing adequate levels of soluble iron. Successful utilization of the pMMO enzyme can then extend to *in vivo* studies (Fig. 7).

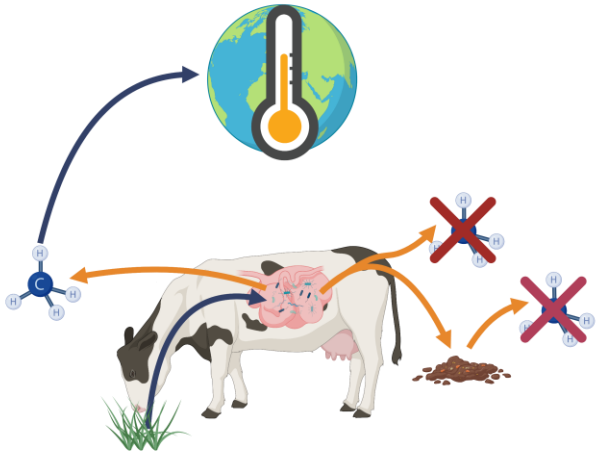

Fig. 7. Reduction of Methane Emissions in Enteric Rumination. Schematic demonstrates the potential reduction of global warming effects by the implementation of modified *E. coli* to oxidize methane from enteric rumination and the subsequent reduction of produced methane emissions.

Broader Impacts

Implementing successfully modified *E. coli* into bovine populations and ensuring no detrimental impacts on bovine health has the potential of reducing methane emissions in the bovine production sector. While anthropogenic methane emissions need to reduce by 45% of current emissions, any reduction in methane emissions from enteric rumination, once extended to large bovine populations, would lead to immediate benefits for human, animal, and environmental given the 12-year atmospheric lifetime of methane.

Materials

Plasmid vectors encoding three subunits of particulate methane monooxygenase (pmoA, pmoB, and pmoC) were

developed and published by GenScript. Each subunit was expressed in three separate pUC57 plasmids containing ampicillin resistance and a LacZ promoter. Luria-Bertani (LB) powder was purchased from Sigma Aldrich and produced in the lab. LB agar plates with 50 µg/mL ampicillin were obtained from Thermo Fisher Scientific. BL21 *E. coli* strains were provided by the Berger Lab within the Department of Chemical Engineering at the University of Virginia.

End Matter

Author Contributions and Notes

C.N.D. and G.P.P. designed research, C.N.D. and A.R.A performed bacterial transformations, C.N.D., A.R.A., and G.N.B. ran validation experiments, C.N.D. wrote data analytics software, C.N.D. performed data analysis; and C.N.D., A.R.A, and G.N.B. wrote the paper. The authors declare no conflict of interest.

Acknowledgments

We graciously thank Madison Mann of the Berger Lab for her time and dedication to our bacterial transformations. Funding was given by the Department of Biomedical Engineering at the University of Virginia. All schematics were designed with BioRender.

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