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EDUCATION AND TRAINING

- **Assistant Research Professor** May 2017- present
  Postdoctoral Scholar Jan. 2015-May 2017
  Advisor: Prof. James Gregory Ferry
  Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, USA
  Research interest: Understanding the biochemistry and physiology of methanogenic archaea

- **Ph.D. (Biochemistry)** Aug. 2009 -Dec. 2014
  Advisor: Prof. Eduardus (Evert) Duin
  Department of Chemistry and Biochemistry, Auburn University, AL, USA
  Dissertation: "Methyl-coenzyme M reductase: Elucidating the process of activation and study of the effect of the methanogenesis inhibitor 3-nitrooxypropanol"

- **Research Assistant (Bio-Electromagnetic laboratory)** Aug. 2008-July 2009
  M. Phil. (Biochemistry/ Environmental Sciences) Aug. 2006- July 2008
  Advisor: Prof. Jitendra Behari
  School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India
  Project: "Synergistic Effect of Hydroxyapatite Nanoparticles and Pulsed Electromagnetic Field to Prevent Osteoporosis in Rat Induced by Simulated Microgravity"

- **M.Sc. (Biotechnology)** July 2004- Jun 2006
  Department of Biotechnology, Goa University, Goa, India
  Project: "Comparative Study on Growth-Substrate Utilized by Marine and Terrestrial Microorganism involved in biodegradation of Xenobiotics**"**

PUBLICATIONS

Online listing of publications:
Google Scholar: (https://scholar.google.com/citations?user=3Wn8-KIAAAAJ&hl=en)

1. **Divya Prakash**, Prashanti R. Iyer, Suharti Suharti, Karim A. Walters, John H. Golbeck, Katsuhiko S. Murakami and James G. Ferry. (2019) "Structure and Function of Flavodoxin from the Domain Archaea Stabilizing an Anionic Semiquinone" ***Proc. Natl. Acad. Sci. U.S.A,*** 116 (51) 25917-25922
2. **Divya Prakash*,** Shikha S. Chauhan*, James G. Ferry. (2019). "Life on the Thermodynamic Edge: Respiratory Growth of an Acetotrophic Methanogen" ***Sci. Adv.*** **5** : eaaw9059 (equal contribution)
   - Pennsylvania State University News: https://science.psu.edu/news/Ferry9-2019
   - ScienceDaily News: https://www.sciencedaily.com/releases/2019/09/190904175713.htm

3. **Prakash, D**., Chauhan, S., & Behari, J. (2019). "Therapeutic effectiveness of Hydroxyapatite Nanoparticles and Pulsed Electromagnetic Field in Osteoporosis and Cancer". ***Journal Of Advances In Biotechnology***, 8, 1058-107
4. **Prakash, D**., Walters, K. A., Martinie, R. J., Mc Carver, A. C., Kumar, A. K., Lessner, D. J., Krebs, C., Golbeck, J. H., and Ferry, J. G. (2018). "Toward a mechanistic and physiological understanding of a ferredoxin: disulfide reductase from the domains Archaea and Bacteria". ***Journal of Biological Chemistry*** 293, 9198-9209
5. Evert C. Duin, Tristan Wagner, Seigo Shima, **Divya Prakash**, Bryan Cronin, David R. Yáñez-Ruiz, Stephane Duval, Robert Rümbeli, René T. Stemmler, Rudolf Kurt Thauer, and Maik Kindermann. (2016). "Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol". ***Proc. Natl. Acad. Sci. U.S.A,*** 113 (22), 6172–6177.
6. **Divya Prakash*,** Deepa Yenugudhati*, Adepu K. Kumar, R. Siva Sai Kumar, Neela H. Yennawar, Hemant P. Yennawar, and James G. Ferry. (2016). "Structural and Biochemical Characterization of Methanoredoxin from *Methanosarcina acetivorans*, a Glutaredoxin-Like Enzyme with Coenzyme M-Dependent Protein Disulfide Reductase Activity". ***Biochemistry***, *55* (2), 313–321. **(* equal contribution)**
7. Yifeng Wei, Bin Li**, Divya Prakash**, James G. Ferry, Sean J. Elliott, and JoAnne Stubbe. (2015). "A Ferredoxin Disulfide Reductase Delivers Electrons to the *Methanosarcina barkeri* Class III Ribonucleotide Reductase". ***Biochemistry***, 54 (47), pp 7019–7028.
8. **Divya Prakash**, Yonnie Yu, Sang-Jin Suh, Evert C Duin (2014). "Elucidating the process of activation of Methyl-Coenzyme M Reductase". ***Journal of Bacteriology***, 196 (13), 2491-98.
9. **Prakash Divya,** J Behari. (2009). "Synergistic role of hydroxyapatite nanoparticles and pulsed electromagnetic field therapy to prevent bone loss in rats following exposure to simulated microgravity". ***International Journal of Nanomedicine*** 4, 133-144.
10. **Divya Prakash**, Rakesh Kumar Roshan, U.M.X Sangodkar, C.U. Rinvorker. (2008). "Isolation and Characterization of meta- toluic acid degrading marine bacterium". ***Indian Journal of Geo-Marine Sciences*** 37(3) 322-325.
11. Duin, E.C., **Prakash, D.**, and Brungess, C. (2011). "Methyl-coenzyme M reductase from *Methanothermobacter marburgensis*" ***Methods of Enzymology***, 494, 159-187.
12. Bhaskarjyoti Bodo, **Divya Prakash**, and P. K. Kalita. (2012). "Synthesis and Characterization of ZnS: Mn Nanoparticles". ***International Journal of Applied Physics and Mathematics***, Vol. 2, No. 3, May 2012.
13. **Prakash Divya,** J. Behari (2008). "Synergistic role of hydroxyapatite nanoparticles and pulsed electromagnetic field therapy to prevent bone loss in rats following exposure to simulated microgravity". ***Proceedings of International Conference on Microwave – 08,*** 572-573.
14. J. Behari, J. Manjhi, **D. Prakash** (2010). "A pulsed electromagnetic field stimulation in fracture healing and in induced osteoporosis in Wistar rats" ***Osteoporosis International***, 21.158

MANUSCRIPTS IN PREPARATION/SUBMITTED

1. Thomas M. Deere, **Divya Prakash**, Faith H. Lessner, Evert C. Duin, and Daniel J. Lessner "A role for IscS and IscU in cysteine dependent iron-sulfur cluster biogenesis in *Methanosarcina acetivorans*" [submitted to ***Applied & Environmental Microbiology***]
2. **Divya Prakash,** James G. Ferry "Proteomics gives insight into the regulatory function of Ferredoxin Thioredoxin Reductase in *Methanosarcina acetivorans*".
3. Thomas Giunta, Jeanine Ash, Jabrane Labidi, Douglas Rumble III, Tina Treude, Sebastian Krause, Rachel Harris, **Divya Prakash**, James Gregory Ferry, Mojhgan Haghnegahdar,

Edwin Schauble, Barbara Sherwood Lollar, Edward D. Young "A Two-Dimensional Perspective on CH_4 Isotope Clumping: Process vs. Source".

4. Michel G.S.Martinez, Jin Xiong, Carly Engel, **Divya Prakash**, Yisong Guo, Evert C. Duin, James G Ferry, "Biochemical and Spectroscopic characterization of Heterodisulfide reductase B2 subunit of *Methanosarcina acetivorans* suggests one-cluster reaction mechanism direct by 4Fe-4S cluster in N-terminal CCG motif.

TEACHING / MENTORING EXPERIENCES

- Graduate Teaching Assistant at the Department of Chemistry and Biochemistry at Auburn University, Auburn (Aug.2009- Aug.2014)
  Labs taught**:** Biochemistry (BCHE 5181, 5191), General Chemistry (CHEM 1031, 1041) and Organic Chemistry (CHEM 2071, 2081).
- Mentored undergraduate students to the successful completion of short term research projects in the lab (Department of Chemistry and Biochemistry at Auburn University, AL, USA (2010-2014).
- Mentored summer training students (2007-2008), School of Environmental Sciences, Jawaharlal Nehru University at New Delhi (India).

OUTREACH

- Judged "2018 Undergraduate Exhibition and 11th Annual Postdoc Research Exhibition" at Penn State University, University Park, USA.

***Editor*:**

- Journal of Advances in Biotechnology (JBT), Council for Innovative Research (CIRWORLD) publishers (https://cirworld.com/index.php/jbt/about/editorialTeam).
- Journal of Experimental Research on Human growth & Aging (JERHA), Medwin Publishers (https://medwinpublishers.com/JERHA/editorial-board.php).
- Archive of Biochemistry, Peertecz Publications Inc. (https://www.peertechz.com/editor/divya-prakash).

Reviewer (https://publons.com/researcher/1303718/divya-prakash/)**:**

- International Journal of Nanomedicine- Dove Medical Press.
- Journal of Advances in Bioscience and Biotechnology (ABB)-Scientific Research Publishing (SCIRP).
- International Journal of Molecular Sciences, Pathogens and Marine Drugs, Sustainability– MDPI publisher.

PROFESSIONAL EXPERIENCES & ACTIVITIES

- May 2017-present: Assistant Research Professor, Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA
- Participated in **3rd Annual Neutron Scattering** 2013 for Novices Workshop at **Oak Ridge National Lab**, Tennessee.
- Aug. 2009 – Dec. 2014: Graduate Teaching/ Research Assistant, Department of Chemistry and Biochemistry, Auburn University, Auburn, AL
- Aug. 2006– July 2008: Junior Research Fellow, School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India.

AWARDS AND FELLOWSHIPS

- Awarded 2014 and 2012 **National Science Foundation Summer Graduate Research Auburn University Cellular and Molecular Biosciences (CMB) Peaks of Excellence Research Fellowship**.

- Awarded for the **Malone-Zallen Graduate Research Fellowship** 2013.
- Awarded travel award 2018 by the office of postdoctoral Association (OPA), Penn State University, University Park, PA.
- 2014 **American Society of Biochemistry and Molecular Biology (ASBMB) graduate / postdoctoral travel award**.
- Student Travel awards, School of Science and Mathematics, Auburn University (2012, 2014).
- Received the second prize for Postal Presentation in Workshop cum Symposium on "Electromagnetic Fields Bio-Interactions, Applications and Mobile Computing: Electromagnetic Pollution", Organized by School of Environmental Sciences, Jawaharlal Nehru University, New Delhi-110067 on March 14-15, 2008.
- Awarded Junior Research Fellowship from Council of Scientific & Industrial Research-University Grants Commission (C.S.I.R-UGC) (India), Dec 2006.
- Qualified in all **India Graduate Aptitude Test (GATE) 2005 (98.72 percentil**e)

TALKS

- Short talk at ***Gordon Research Conference*** **on Applied and Environmental Microbiology (Shaping the Earth's Microverse)** held July 14,2019-July 19, 2019 at Mount Holyoke College South Hadley, MA, US
- Short talk at ***Gordon Research Conference*** **on Molecular Basis of Microbial One-Carbon Metabolism (Dynamic One-Carbon Use on a Changing Planet)** held July 29, 2018 - August 03, 2018, at Grand Summit Hotel at Sunday River in Newry, ME United States.
- **Invited speaker for Molecular Basis of Microbial One –Carbon Metabolism, *Gordon-Kenan Research Seminar* 2012,** "Isolation and characterization of protein components required for activation of methyl coenzyme M reductase"**,** August 4-5, 2012 at Lewiston, ME, US
- **ARPA-E REMOTE Program: Archaeal Conversion of Methane to Liquid Fuels at Pennsylvania State University,** October 30, 2015, and April 12, 2016. "Understanding Biological Methane Oxidation Using Methyl-Coenzyme M Reductase from *M. acetivorans* and ANME-1"
- Department of Chemistry at the Massachusetts Institute of Technology, July 2014 "Elucidating the process of activation of Methyl-Coenzyme M Reductase"
- Department of Microbiology at the University of Washington, Seattle, August 2014, "Elucidating the process of activation of Methyl-Coenzyme M Reductase"
- Division of Developmental Biology at Cincinnati Children's Hospital June 2014, "Elucidating the process of activation of Methyl-Coenzyme M Reductase"
- "Elucidating the process of activation of Methyl-Coenzyme M Reductase" **99th Annual meeting of Southeastern Branch of ASM,** November 7-9, 2013 at Auburn University Hotel and Conference Center, Auburn, AL, USA.

POSTER PRESENTATIONS

- **Divya Prakash**, Shikha S. Chauhan & James G. Ferry "Re-assessing the roles of methanogens and Fe(III) in anaerobic microbial networks of coastal marine sediments" ***BacNet19 conference*** held at the Hotel Eden Roc, in Sant Feliu de Guíxols SPAIN September 1-6, 2019
- **Divya Prakash**, Shikha S. Chauhan & James G. Ferry " Re-assessing the Roles of Methanogens and Fe(III) in Anaerobic Microbial Food Chains of Coastal Marine Sediments" ***Gordon Research Conference*** **on Applied and Environmental Microbiology (Shaping the Earth's Microverse)** held July 14,2019-July 19, 2019 at Mount Holyoke College South Hadley, MA, US
- **Divya Prakash**, Prashanti R. Iyer, Suharti Suharti, Zhen Yan, Karim Walters, John Golbeck,

Katsuhiko Murakami & James G. Ferry, "Structure and function of an unusual flavodoxin from the domain Archaea" ***Gordon Research Conference*** **on Molecular Basis of Microbial One-Carbon Metabolism (Dynamic One-Carbon Use on a Changing Planet)** held July 29, 2018 - August 03, 2018, at Grand Summit Hotel at Sunday River in Newry, ME United States.

- **Divya Prakash**, Karim A. Walters, Ryan J. Martinie, Addison C. Mc Carver, Daniel J. Lessner, Carsten Krebs, John H. Golbeck & James G. Ferry, "Towards a mechanistic and physiological understanding of the ferredoxin: disulfide reductase from the domains Archaea and Bacteria" ***Gordon Research seminar*** **on Molecular Basis of Microbial One-Carbon Metabolism (C1 Metabolism: From Fundamental Discoveries to Their Biotechnological Applications)** held **July 28 - 29, 2018** at Grand Summit Hotel at Sunday River in Newry, ME United States.
- **Divya Prakash**, Karim A. Walters, Ryan J. Martinie, Addison C. McCarver, Daniel J. Lessner, Carsten Krebs, John H. Golbeck & James G. Ferry, "Towards a mechanistic and physiological understanding of the ferredoxin: disulfide reductase from the domains Archaea and Bacteria" ***2018 Frontiers in Metallobiochemistry Symposium*** at The Pennsylvania State University, University Park Campus, **June 6 – 8, 2018.**
- James G. Ferry, Costas D. Maranas & Thomas K. Wood, **"**Engineering a Methane-to-Acetate Pathway" ***ARPA-E REMOTE PROGRAM KICKOFF*** on February 15-16, 2017, at the Marriott West Loop – Galleria, Houston, TX.
- James G. Ferry, Costas D. Maranas & Thomas K. Wood, **"**Engineering a Methane-to-Acetate Pathway" ***ARPA-E REMOTE PROGRAM KICKOFF*** on January 20-21, 2016, at the Sheraton LaJolla in LaJolla, CA (Presented by **Divya Prakash** as a group member).
- **Divya Prakash**, Sang-Jin Suh, Evert Duin, **"**Elucidating the process of activation of Methyl-Coenzyme M Reductase" **Molecular Basis of Microbial One-Carbon Metabolism**, ***Gorden Research Conference***, August 10-15, 2014, at South Hadley, MA, USA.
- **Divya Prakash**, Sang-Jin Suh, Evert Duin, **"**Elucidating the process of activation of Methyl-Coenzyme M Reductase" **Experimental Biology & American Society for Biochemistry and Molecular Biology,** April 26-30, 2014 at the San Diego Convention Center, San Diego, CA, USA.
- **Divya Prakash**, Evert Duin, **"**Elucidating the process of activation of Methyl-Coenzyme M Reductase from *Methanothermobacter marburgensis",* ***fourth southeast Enzyme Conference***, April 20, 2013, at Georgia State University in Atlanta, Georgia, USA.
- **Divya Prakash**, Evert Duin, "Isolation and characterization of protein components required for activation of methyl coenzyme M reductase, Molecular Basis of Microbial One –Carbon Metabolism" ***Gordon Research Conference***, August 5-10, 2012 at Lewiston, ME, US.
- **Divya Prakash**, Evert Duin, " Activation of Methyl-Coenzyme M Reductase". Archaea: Ecology, Metabolism & Molecular Biology, ***Gordon Research Conference***, July 2011 at Waterville Valley, NH.
- **Divya Prakash**, J.Behari,"Synergistic role of Hydroxyapatite Nanoparticles and Pulsed Electromagnetic Field Therapy to prevent Bone loss in rats following exposed to Simulated Microgravity" ***Proceeding of International Conference on Microwave-08, IEEE***, 572-573.
- **Divya Prakash,** Bhaskarjyoti Bodo, Behari J "Intervention to prevent bone loss in simulated microgravity", Electromagnetic Fields Bio-Interactions, Applications, and Mobile Computing: Electromagnetic Pollution, March 14-15, 2008.

REFERENCES

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   Stanley Person Professor of Biochemistry and Molecular Biology
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   Professor, Department of Chemistry and Biochemistry
   Auburn University, Auburn, AL 36849
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3. Dr. Sang-Jin Suh (Ph.D. thesis committee member)
   Associate Professor, Department of Biomedical Sciences
   Texas A&M College of Dentistry, Dallas, TX 75246
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4. Dr. Doug Goodwin (Ph.D. thesis committee member)
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ECOLOGY

Life on the thermodynamic edge: Respiratory growth of an acetotrophic methanogen

Divya Prakash*, Shikha S. Chauhan*, James G. Ferry†

Although two-thirds of the nearly 1 billion metric tons of methane produced annually in Earth's biosphere derives from acetate, the in situ process has escaped rigorous understanding. The unresolved question concerns the mechanism by which the exceptionally marginal amount of available energy supports acetotrophic growth of methanogenic archaea in the environment. Here, we show that *Methanosarcina acetivorans* conserves energy by Fe(III)-dependent respiratory metabolism of acetate, augmenting production of the greenhouse gas methane. An extensively revised, ecologically relevant, biochemical pathway for acetotrophic growth is presented, in which the conservation of respiratory energy is maximized by electron bifurcation, a previously unknown mechanism of biological energy coupling. The results transform the ecological and biochemical understanding of methanogenesis and the role of iron in the mineralization of organic matter in anaerobic environments.

INTRODUCTION

Methanogenic archaea, methanogens, are essential players in the global carbon cycle, producing 1 billion metric tons of methane annually, of which two-thirds derives from the methyl group of acetate by acetotrophic species. They are terminal organisms of microbial food chains that decompose biomass to methane in Earth's diverse anaerobic biospheres. As methane accounts for about 30% of net anthropogenic radiative forcing, acetotrophic methanogens are accountable for a significant share of the global warming that affects climate change (*1*). Accordingly, it is proposed that the evolution of efficient acetotrophic methanogens produced a methanogenic burst in the end-Permian carbon cycle that contributed to a sharp increase in global warming and Earth's greatest mass extinction (*2*).

Acetotrophic methanogens are important players in Earth's ecology, yet their role is fragile, considering that they live on the thermodynamic edge (*3*, *4*). On the basis of present understanding, it is not abundantly clear how acetotrophic methanogens conserve enough energy for growth under ideal laboratory conditions, let alone in competitive environments (*5*). The current acetoclastic pathway relies strictly on a fermentative mode, where the carbonyl group of acetate is oxidized with electrons transferred to the endogenous electron-accepting methyl group that produces methane (*4*). The net free energy available for growth is exceedingly small considering the requirement for activation of acetate by adenosine triphosphate (ATP) (Eq. 1) (*3*). The energy available by fermentative

$$CH_3CO_2H\,(aq) \rightarrow CO_2(g) + CH_4(g)\ \Delta G^{\circ\prime} = -36\ kJ/mol \quad (1)$$

methanogenesis is in stark contrast to acetotrophic nonmethanogenic anaerobes from the domain *Bacteria* that use exogenous electron acceptors such as ferric iron for respiration (Eq. 2) (*6*).

$$CH_3COO^- + 24Fe(OH)_3(s)\ (ferrihydrite) \rightarrow 8\,Fe_3O_4(s)\ (magnetite) + 2\,HCO_3^- + 36\,H_2O + H^+ \quad \Delta G^{\circ\prime} = -707\ kJ/mol \quad (2)$$

The possibility of respiratory-driven acetotrophic growth of *Methanosarcina acetivorans* was advanced by the recent finding that membrane vesicles from acetate-grown cells generate a Na^+ gradient that drives ATP synthesis dependent on the oxidation of ferredoxin (Fdx) and the reduction of Fe(III) citrate (*7*). Furthermore, the effects of ferrihydrite amendment on the population dynamics of methanogens in rice field soil slurries support the proposal that rice cluster 1 (RC-1) methanogens may conserve energy by reducing Fe(III) (*8*). Notably, methanogens are of ancient origin, and Fe(III) reduction has been suggested to be of importance in the early evolution of respiration (*9*). However, Fe(III)-dependent respiratory growth has not been reported for any methanogenic pathway. Here, we show respiratory metabolism of acetate by *M. acetivorans* dependent on the reduction of ferrihydrite that produces a twofold increase in growth and augments production of the greenhouse gas methane. A revised, ecologically relevant, biochemical pathway is proposed, where electron bifurcation (EB) maximizes the conservation of respiratory energy. EB is a recently discovered mechanism of energy conservation in anaerobic microbes, where an electron pair is bifurcated with transfer of one electron to an acceptor with a more negative redox potential driven by transfer of the second electron to an acceptor with a more positive potential (*5*). The results document respiratory energy conservation previously unrecognized for any methanogenic species, which transforms the ecological and biochemical understanding of methanogenesis and the role of iron in anaerobic environments.

RESULTS

An investigation of respiratory-driven acetotrophic growth of *M. acetivorans* was prompted by the previous finding that everted membrane vesicles from acetate-grown cells generate a Na^+ gradient dependent on the reduction of Fe(III) (*7*). Acetotrophic growth, gaged by protein production (Fig. 1A), was enhanced by supplementing the medium with 20 mM ferrihydrite. Growth with ferrihydrite was twofold greater when the medium was further supplemented with 0.2 mM anthraquinone-2,6-disulfonate (AQDS), in which the trend was reflected in cell counts (Fig. 2). The low concentration of added AQDS was intended only as a mediator of electron transport to focus on the more ecologically relevant ferrihydrite. This concentration of AQDS had no individual effect on the growth parameters (Fig. 1). Results obtained for cultures amended with ferrihydrite

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Structure and function of an unusual flavodoxin from the domain *Archaea*

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Edited by Mary E. Lidstrom, University of Washington, Seattle, WA, and approved November 11, 2019 (received for review May 21, 2019)

Flavodoxins, electron transfer proteins essential for diverse metabolisms in microbes from the domain *Bacteria*, are extensively characterized. Remarkably, although genomic annotations of flavodoxins are widespread in microbes from the domain *Archaea*, none have been isolated and characterized. Herein is described the structural, biochemical, and physiological characterization of an unusual flavodoxin (FldA) from *Methanosarcina acetivorans*, an acetate-utilizing methane-producing microbe of the domain *Archaea*. In contrast to all flavodoxins, FldA is homodimeric, markedly less acidic, and stabilizes an anionic semiquinone. The crystal structure reveals an flavin mononucleotide (FMN) binding site unique from all other flavodoxins that provides a rationale for stabilization of the anionic semiquinone and a remarkably low reduction potentials for both the oxidized/semiquinone (−301 mV) and semiquinone/hydroquinone couples (−464 mV). FldA is up-regulated in acetate-grown versus methanol-grown cells and shown here to substitute for ferredoxin in mediating the transfer of low potential electrons from the carbonyl of acetate to the membrane-bound electron transport chain that generates ion gradients driving ATP synthesis. FldA offers potential advantages over ferredoxin by (*i*) sparing iron for abundant iron-sulfur proteins essential for acetotrophic growth and (*ii*) resilience to oxidative damage.

electron transport | methanogenesis | anaerobic | greenhouse gas | global warming

Flavodoxins (Fld) are electron-transfer proteins essential for diverse metabolisms in microbes from the domain *Bacteria*, whereas organisms from the domain *Eukarya* contain multidomain flavoproteins evolved from ancestral flavodoxin genes (1, 2). Flds were discovered over 50 y ago in Cyanobacteria and Clostridia growing in low-iron conditions where they serve as electron carriers in enzyme systems operating at potentials near that of the hydrogen electrode (3, 4). Flds contain 1 molecule of redox active flavin mononucleotide (FMN) that is noncovalently bound. All Flds characterized are highly acidic proteins containing between 140 and 180 residues that are divided into short-chain and long-chain types differing by a 20-residue loop of yet unknown function (2). The protein environment of FMN stabilizes the neutral form of the semiquinone (sq), producing dramatic shifts in the reduction potentials for each of 2 1-electron reductions of the flavin. The FMN of Flds cycle between the sq and hydroquinone (hq) for which the reduction potentials are below −101 mV for free flavin (5, 6).

Genome annotations for Flds are widespread among microbes in the domain *Archaea*. Although characterizations of Flds from the domain *Bacteria* are abundant, none from *Archaea* are reported. Metabolically diverse species of the domain *Archaea* are present in a variety of environments where they play major roles in the biogeochemical cycling of carbon, nitrogen, and sulfur. Methane-producing species (methanogens) are the largest group in the domain for which annotations of genomes include an abundance of Flds (https://www.ncbi.nlm.nih.gov/pubmed) not yet investigated. Methanogens are terminal organisms of microbial food chains that decompose plant biomass to methane in Earth's diverse anaerobic environments. The process produces an annual 10^9 metric tons of methane of which two-thirds derives from the methyl group of acetate by acetotrophic species. As atmospheric methane accounts for about 30% of net anthropogenic radiative forcing, acetotrophic methanogens are responsible for a substantial share of global warming and adverse climate change (7). The evolution of efficient acetotrophic methanogens, represented by *Methanosarcina acetivorans*, is proposed to have produced a methanogenic burst in the end-Permian carbon cycle, contributing to a dramatic increase in global warming and Earth's greatest mass extinction (8).

M. acetivorans has emerged as a model for understanding the biochemistry of acetotrophic *Methanosarcina* species. In the acetoclastic pathway, the C-C bond of acetate is cleaved by the CO dehydrogenase/acetyl-CoA decarbonylase (CODH/ACD), yielding a methyl group that is reduced to methane with electrons derived from oxidation of the carbonyl group to CO_2 that is also catalyzed by the CODH/ACD (9). Electrons are channeled through a membrane-bound electron transport chain that begins with the Rnf complex and generates sodium and proton gradients driving ATP synthesis (10–13). Initially guided by findings that ferredoxin (Fdx) is an electron donor to Rnf homologs in the domain *Bacteria*, a Fdx isolated from *M. acetivorans* was shown to

Significance

A flavodoxin from the domain *Archaea* has been characterized. It is the first of any flavodoxin shown to stabilize an anionic semiquinone, providing a platform for understanding how the protein environment modulates the reduction potentials of flavins. The unusual flavodoxin plays an electron transport role in the pathway of acetate conversion to methane in *Methanosarcina acetivorans*, a model methanogen for investigating the process by which two-thirds of the 1 billion metric tons of methane are produced annually in Earth's anaerobic biospheres with a substantial contribution to global warming effecting climate change. Homologs of the gene encoding the flavodoxin are uniformly distributed in diverse acetotrophic methanogens consistent with a wider range of electron transport functions awaiting discovery.

Author contributions: K.S.M. and J.G.F. designed research; D.P., P.R.I., S.S., K.A.W., and K.S.M. performed research; M.G.S.-M. contributed new reagents/analytic tools; D.P., P.R.I., S.S., K.A.W., J.H.G., K.S.M., and J.G.F. analyzed data; and J.H.G., K.S.M., and J.G.F. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

Data deposition: The X-ray crystal structure coordinates and structure factors have been deposited in the Protein Data Bank (ID code 5WID).

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1908578116/-/DCSupplemental.

First published December 4, 2019.

Toward a mechanistic and physiological understanding of a ferredoxin:disulfide reductase from the domains Archaea and Bacteria

Received for publication, February 15, 2018, and in revised form, April 24, 2018 Published, Papers in Press, May 2, 2018, DOI 10.1074/jbc.RA118.002473

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Disulfide reductases reduce other proteins and are critically important for cellular redox signaling and homeostasis. *Methanosarcina acetivorans* is a methane-producing microbe from the domain Archaea that produces a ferredoxin:disulfide reductase (FDR) for which the crystal structure has been reported, yet its biochemical mechanism and physiological substrates are unknown. FDR and the extensively characterized plant-type ferredoxin:thioredoxin reductase (FTR) belong to a distinct class of disulfide reductases that contain a unique active-site [4Fe-4S] cluster. The results reported here support a mechanism for FDR similar to that reported for FTR with notable exceptions. Unlike FTR, FDR contains a rubredoxin [1Fe-0S] center postulated to mediate electron transfer from ferredoxin to the active-site [4Fe-4S] cluster. UV-visible, EPR, and Mössbauer spectroscopic data indicated that two-electron reduction of the active-site disulfide in FDR involves a one-electron-reduced $[4Fe\text{-}4S]^{1+}$ intermediate previously hypothesized for FTR. Our results support a role for an active-site tyrosine in FDR that occupies the equivalent position of an essential histidine in the active site of FTR. Of note, one of seven Trxs encoded in the genome (Trx5) and methanoredoxin, a glutaredoxin-like enzyme from *M. acetivorans*, were reduced by FDR, advancing the physiological understanding of FDR's role in the redox metabolism of methanoarchaea. Finally, bioinformatics analyses show that FDR homologs are widespread in diverse microbes from the domain Bacteria.

Disulfide reductases are universal in nature, where they interact with protein and small molecule substrates required for redox control and response to oxidative stress. Disulfide reductases include thioredoxin reductase (TrxR),[7] an NAD(P)H-dependent flavoenzyme containing an active-site disulfide that transfers reducing equivalents from the flavin to the disulfide of thioredoxin (Trx) by a disulfide-exchange mechanism (1, 2). However, plant ferredoxin:thioredoxin reductase (FTR) is devoid of flavin and contains a novel active-site $[Fe_4S_4]$ cluster (3, 4). *Methanosarcina acetivorans*, classified in the domain Archaea, produces a plant-type ferredoxin:disulfide reductase (FDR) also devoid of flavin that contains an active-site $[Fe_4S_4]$ cluster revealed by the crystal structure (5). FDR is representative of a diverse family of disulfide reductases proposed to have evolved from an ancestral plant-type FTR catalytic subunit to meet a variety of specific ecological needs (6, 37).

Preliminary characterization of FDR involved ferredoxin (Fdx)-dependent reduction of GSSG, a nonphysiological substrate absent in methane-producing species from the domain Archaea (methanoarchaea) (5). Although FDR homologs are widely distributed among diverse methanoarchaea, the physiological redox substrates are unknown (6, 7). Trx is a potential physiological substrate for FDR from *M. acetivorans*, as it encodes seven Trx homologs (Trx1–Trx7) and a NADPH-dependent TrxR shown to reduce only Trx7 (8). However, NADP is not a primary electron carrier in methanoarchaea. Instead, the primary electron carriers are coenzyme F_{420} (F_{420}) and Fdx, underscoring the physiological importance of the Fdx-dependent FDR.

Trxs are small redox proteins that reduce the disulfide bonds of proteins that are key to controlling a diverse array of essential processes in organisms from the domains Bacteria and Eukarya (1). Remarkably, little is known of the biochemistry and physi-

The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Tables S1 and S2 and Figs. S1–S7.

[1] Supported by National Science Foundation Graduate Research Fellowship Program Grant DGE1255832.

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[4] Supported by NASA Exobiology Grant NNX12AR60G and the Arkansas Biosciences Institute, the major research component of the Arkansas Tobacco Settlement Proceeds Act of 2000.

[5] Supported by National Science Foundation Grants MCB-1613022 and MCB-1359578 for the EPR spectroscopy and redox titrations, respectively.

[6] Supported by the Division of Chemical Sciences, Geosciences and Biosciences, Office of Basic Energy Sciences of the United States Department of Energy through Grants DE-FG02-95ER20198 MOD16 and 17 (90%) and the Person Endowment (10%). To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, Pennsylvania 16802. Tel.: 814-863-5721; E-mail: jgf3@psu.edu.

[7] The abbreviations used are: TrxR, thioredoxin reductase; FDR, ferredoxin disulfide reductase; FTR, ferredoxin thioredoxin reductase; NEM, *N*-ethylmaleimide; NEM-WT, NEM-modified WT FDR; Trx, thioredoxin; HSCoM, coenzyme M; HSCoB, coenzyme B; FNR, ferredoxin-$NADP^+$ reductase; F_{420}, coenzyme F_{420}; Fdx, ferredoxin; T, tesla(s); FNR, Fdx-$NADP^+$ reductase; MRX, methanoredoxin.

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Elucidating the Process of Activation of Methyl-Coenzyme M Reductase

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Methyl-coenzyme M reductase (MCR) catalyzes the reversible reduction of methyl-coenzyme M (CH_3-S-CoM) and coenzyme B (HS-CoB) to methane and heterodisulfide CoM-S-S-CoB (HDS). MCR contains the hydroporphinoid nickel complex coenzyme F_{430} in its active site, and the Ni center has to be in its Ni(I) valence state for the enzyme to be active. Until now, no *in vitro* method that fully converted the inactive MCR_{silent}-Ni(II) form to the active MCR_{red1}-Ni(I) form has been described. With the potential use of recombinant MCR in the production of biofuels and the need to better understand this enzyme and its activation process, we studied its activation under nonturnover conditions and achieved full MCR activation in the presence of dithiothreitol and protein components A2, an ATP carrier, and A3a. It was found that the presence of HDS promotes the inactivation of MCR_{red1}, which makes it essential that the activation process is isolated from the methane formation assay, which tends to result in minimal activation rates. Component A3a is a multienzyme complex that includes the *mcrC* gene product, an Fe-protein homolog, an iron-sulfur flavoprotein, and protein components involved in electron bifurcation. A hypothetical model for the cellular activation process of MCR is presented.

The highest heat of combustion per mass unit (55.7 kJ/g) among all the hydrocarbons makes methane an important source of clean renewable energy (1). At the same time, it is also a potent greenhouse gas, with surplus methane production in the geological cycle leading to a slow increase in atmospheric methane concentrations. Understanding the mechanism of methane formation and activation is important for controlling methane emission and improvement of the production of alternative fuels in the form of natural gas. Methyl-coenzyme M reductase (MCR) is the central enzyme in biological methane formation by methanogenic *Archaea* and consumption by anaerobic methanotrophs (2–4). For the methane production, it catalyzes the exergonic conversion of methyl coenzyme M (CH_3-S-CoM) and coenzyme B (HS-CoB) into methane and the heterodisulfide of coenzyme B and coenzyme M (CoB-S-S-CoM [HDS]):

$$CH_3\text{-S-CoM} + \text{H-S-CoB} \rightleftarrows CH_4 + \text{CoM-S-S-CoB}, \quad \Delta G^{O\prime} = -30\ \text{kJ} \cdot \text{mol}^{-1}$$

MCR is composed of three different subunits, α, β, and γ, forming an $(\alpha\beta\gamma)_2$ heterohexamer (5). Each molecule of MCR contains two molecules of the cofactor F_{430}, a Ni-porphinoid, as a prosthetic group (6–8). The binding sites of two coenzymes F_{430} are roughly 50 Å apart, forming two separated structurally identical active sites. The active-site region of MCR contains five post-translationally modified (PTM) amino acids (5, 9) comprising 1-N-methyl-Hisα^{257}, S-methyl-Cysα^{452}, 5-methyl-Argα^{71}, and 2-methyl-Glnα^{400}, and thio-Glyα^{445}. The role of these modifications is not known. In addition, MCR has been subjected to numerous spectroscopic and crystallographic studies. Despite this, the details of the catalytic mechanism are still not clear (10–13).

It is well established that the MCR_{red1}-Ni(I) form is active, while the other two major forms that can be detected in purified enzyme and whole cells, MCR_{ox1}-Ni(III) and MCR_{silent}-Ni(II), are inactive (14–18). The MCR_{red1} form can be obtained by incubating *Methanothermobacter marburgensis* cells either with H_2 or CO (15, 19); however, it is extremely O_2 sensitive due to the low midpoint potential of the Ni(II)/Ni(I) couple in F_{430}, which is estimated to be around −650 mV (versus a hydrogen electrode; pH 7) (20). Moreover, the MCR_{ox1} form can be converted to the MCR_{red1} form by incubating it with the reductant Ti(III) citrate (17). In order to study the reaction mechanism or to explore the role of the PTMs, it is important to develop a cell-free system capable of activating inactive MCR. No cell-free system that can fully convert MCR_{silent} to MCR_{red1} has been reported to date. Small amounts of activation, however, were reported using cellular components purified directly from cell extract using column chromatography (21, 22). In the first fractionation step, three fractions were discovered: A, an unknown fraction; B, which turned out to be HS-CoB; and C, MCR itself. Fraction A was further fractionated and contained A1, an F_{420}-reducing hydrogenase; A2, an ATP-binding protein; A3a, an uncharacterized iron-containing protein; and A3b, a methyl-viologen-reducing (F_{420}-nonreducing) hydrogenase. The activation of MCR was measured by detecting methane formation in the headspace of a closed bottle containing a solution with Ti(III) citrate, component A2, MCR, the column fraction containing A3a, ATP, CH_3-S-CoM, and HS-CoB. A maximum activity of ~0.1 μmol min^{-1} mg^{-1} was obtained (21–23). Since then, methods have been established to preserve the active form of MCR during cell harvesting, preparation of cell extract, and subsequent protein purification procedures (14–18). In this case, enzyme can be obtained with a specific activity of up to 100 μmol min^{-1} mg^{-1} (17). The low activity obtained upon activation makes it clear that the activation process is not well understood. It is possible that the small amount of activation was due to the presence of traces of MCR_{ox1} that can be activated in the presence of Ti(III) citrate. It is also possible that

Received 19 March 2014 Accepted 23 April 2014
Published ahead of print 25 April 2014
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doi:10.1128/JB.01658-14

Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol

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Edited by Lonnie O. Ingram, University of Florida, Gainesville, FL, and approved March 30, 2016 (received for review January 8, 2016)

Ruminants, such as cows, sheep, and goats, predominantly ferment in their rumen plant material to acetate, propionate, butyrate, CO_2, and methane. Whereas the short fatty acids are absorbed and metabolized by the animals, the greenhouse gas methane escapes via eructation and breathing of the animals into the atmosphere. Along with the methane, up to 12% of the gross energy content of the feedstock is lost. Therefore, our recent report has raised interest in 3-nitrooxypropanol (3-NOP), which when added to the feed of ruminants in milligram amounts persistently reduces enteric methane emissions from livestock without apparent negative side effects [Hristov AN, et al. (2015) *Proc Natl Acad Sci USA* 112(34):10663–10668]. We now show with the aid of in silico, in vitro, and in vivo experiments that 3-NOP specifically targets methyl-coenzyme M reductase (MCR). The nickel enzyme, which is only active when its Ni ion is in the +1 oxidation state, catalyzes the methane-forming step in the rumen fermentation. Molecular docking suggested that 3-NOP preferably binds into the active site of MCR in a pose that places its reducible nitrate group in electron transfer distance to Ni(I). With purified MCR, we found that 3-NOP indeed inactivates MCR at micromolar concentrations by oxidation of its active site Ni(I). Concomitantly, the nitrate ester is reduced to nitrite, which also inactivates MCR at micromolar concentrations by oxidation of Ni(I). Using pure cultures, 3-NOP is demonstrated to inhibit growth of methanogenic archaea at concentrations that do not affect the growth of nonmethanogenic bacteria in the rumen.

methanogenesis | methyl-coenzyme M reductase | enteric methane | greenhouse gas | climate change

Since the agricultural and industrial revolution 200 y ago, the methane concentration in the atmosphere has increased from less than 0.6 to 1.8 ppm. The present concentration is only 0.45% of that of CO_2, but because methane has a 28- to 34-fold higher global warming potential than CO_2 on a 100-y horizon, it contributes significantly to global warming (1). On the other hand, the lifetime of atmospheric methane is relatively short relative to CO_2. Accordingly, the climate response to reductions of methane emissions will be relatively rapid. Thus, measures targeting methane emissions are considered paramount to mitigate climate change (2).

One of the main anthropogenic sources of atmospheric methane are ruminants (cattle, sheep, goats), the number of which has grown in parallel with the world population. Presently, there are about 1.5 billion cattle, 1.1 billion sheep, and 0.9 billion goats raised by humans (3). Ruminants emit about 100 million tons of methane per year, which corresponds to ∼20% of global methane emissions (4).

In the rumen (Fig. 1), plant material is fermented by anaerobic bacteria, protozoa, fungi, and methanogenic archaea in a trophic chain, predominantly yielding acetate, propionate, butyrate, CO_2, and methane with H_2 as intermediate (5, 6). Whereas organic acids are absorbed and metabolized by the animals, methane escapes the rumen into the atmosphere via eructation and breathing of the animals. The generation of methane by methanogenic archaea in the intestine of domestic ruminants lessens feed efficacy, as up to 12% of the gross energy ingested by the animal is lost this way (7).

Methane (CH_4) formation is the main H_2 sink in the rumen. It is formed by methanogenic archaea at the bottom of the trophic chain mainly from carbon dioxide (CO_2) and hydrogen (H_2) (Fig. 1). However, the methane eructated by ruminants contains only minute amounts of H_2; the concentration of dissolved H_2 in the rumen is near 1 μM (8), equivalent to a H_2 partial pressure of near 140 Pa. Because at 1 μM, H_2 formation from most substrates in the rumen is exergonic (9), the low H_2 concentration indicates that H_2 is consumed in the rumen by the methanogens more rapidly than it is formed by other microorganisms (10). The H_2 concentration increases substantially only when methane formation from H_2 and CO_2 is specifically inhibited by more than 50% (10, 11). Already a small increase in the H_2 concentration (8) leads to both down-regulation of H_2-generating pathways (12) and up-regulation of H_2-neutral and H_2-consuming pathways such as propionate formation, resulting in additional energy

Significance

Methane emission from the ruminant livestock sector—a by-product from enteric fermentation of plant biomass in the ruminant digestive system—is produced by methanogenic archaea and represents not only a significant amount of anthropogenic greenhouse gases contributing to climate change but also an energy loss and a reduction in feed efficacy. The present study elucidates the development and the unique mode of action of the highly specific inhibitor 3-nitrooxypropanol (3-NOP), which is targeting the nickel enzyme methyl-coenzyme M reductase in rumen archaea that catalyzes the methane-forming reaction. At the very low effective concentrations recently applied in vivo (dairy and beef cattle), 3-NOP appears to inhibit only methanogens and thus to be attractive for development as a feed supplement.

Author contributions: E.C.D., D.R.Y.-R., S.D., R.T.S., R.K.T., and M.K. designed research; E.C.D., T.W., S.S., D.P., B.C., D.R.Y.-R., S.D., R.R., and R.T.S. performed research; E.C.D., S.S., R.T.S., R.K.T., and M.K. analyzed data; and R.K.T. and M.K. wrote the paper.

Conflict of interest statement: The authors working at Auburn University and at the Max Planck Institute in Marburg (E.C.D., T.W., S.S., D.P., B.C., and R.K.T.) declare no competing financial interests. The authors affiliated with Estation Experimental del Zaidin (D.R.Y.-R.) or DSM Nutritional Products (S.D., R.R., R.T.S., and M.K.) have filed patent applications for nitrate esters as inhibitors of rumen methanogenesis.

This article is a PNAS Direct Submission.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 5G0R).

See Commentary on page 6100.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1600298113/-/DCSupplemental.