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Electrons to molecules by engineering and evolution: biological upgrading of formate by *Cupriavidus necator*

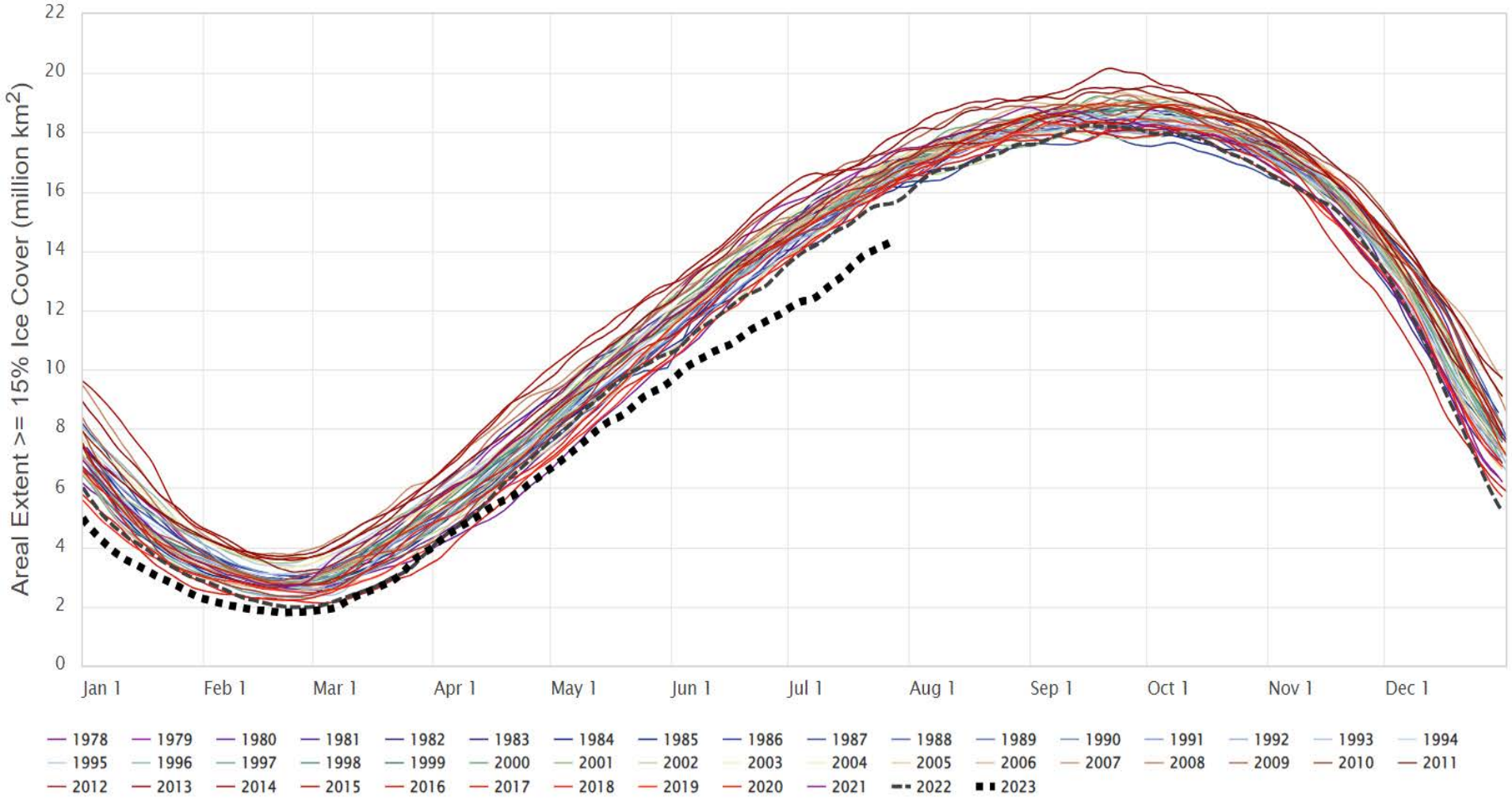
8/1/2023

Chris Calvey

National Renewable Energy Laboratory

We have a problem…

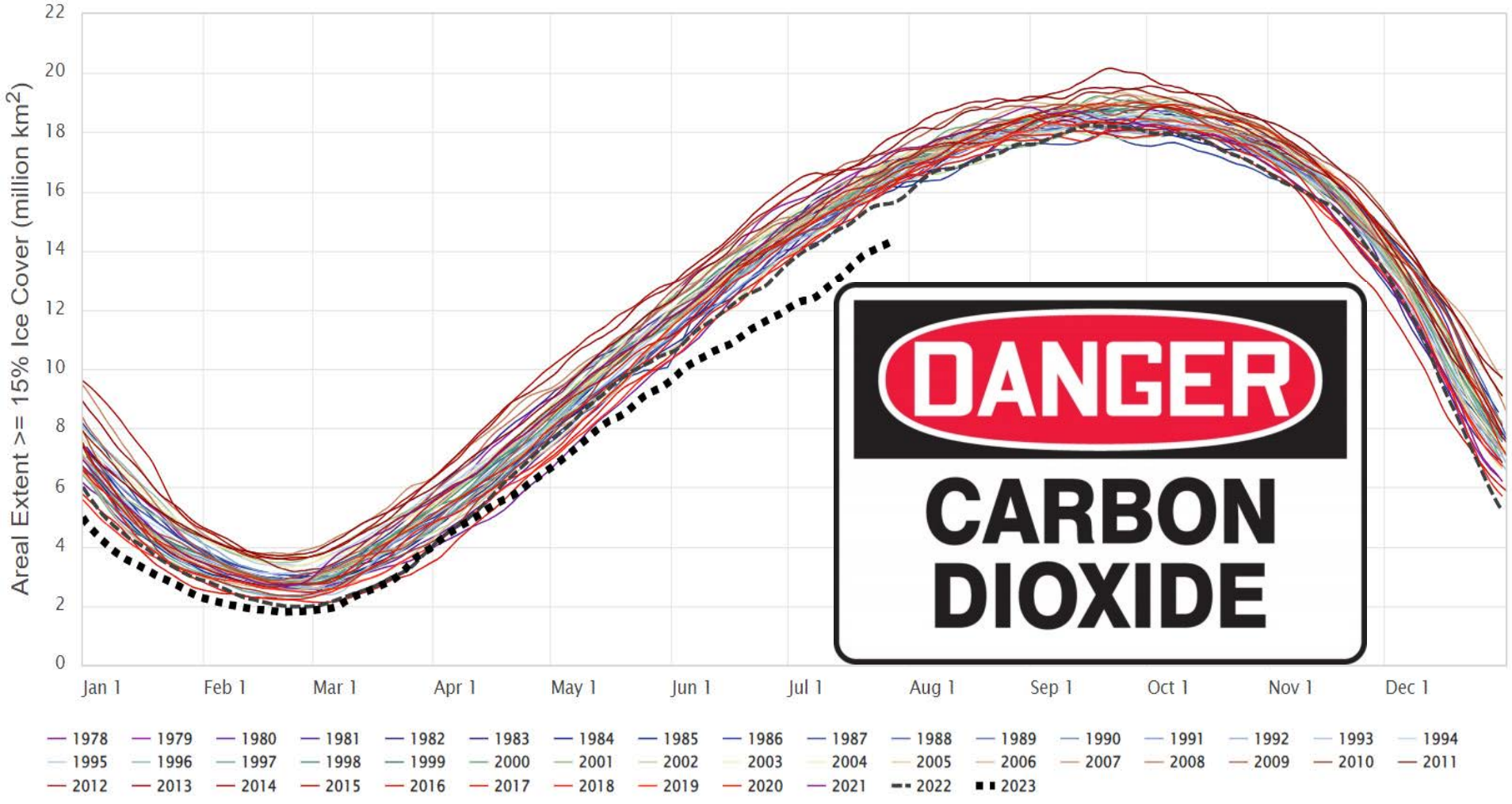
Southern Hemisphere Sea Ice Extent, climatereanalyzer.org



We have a problem…

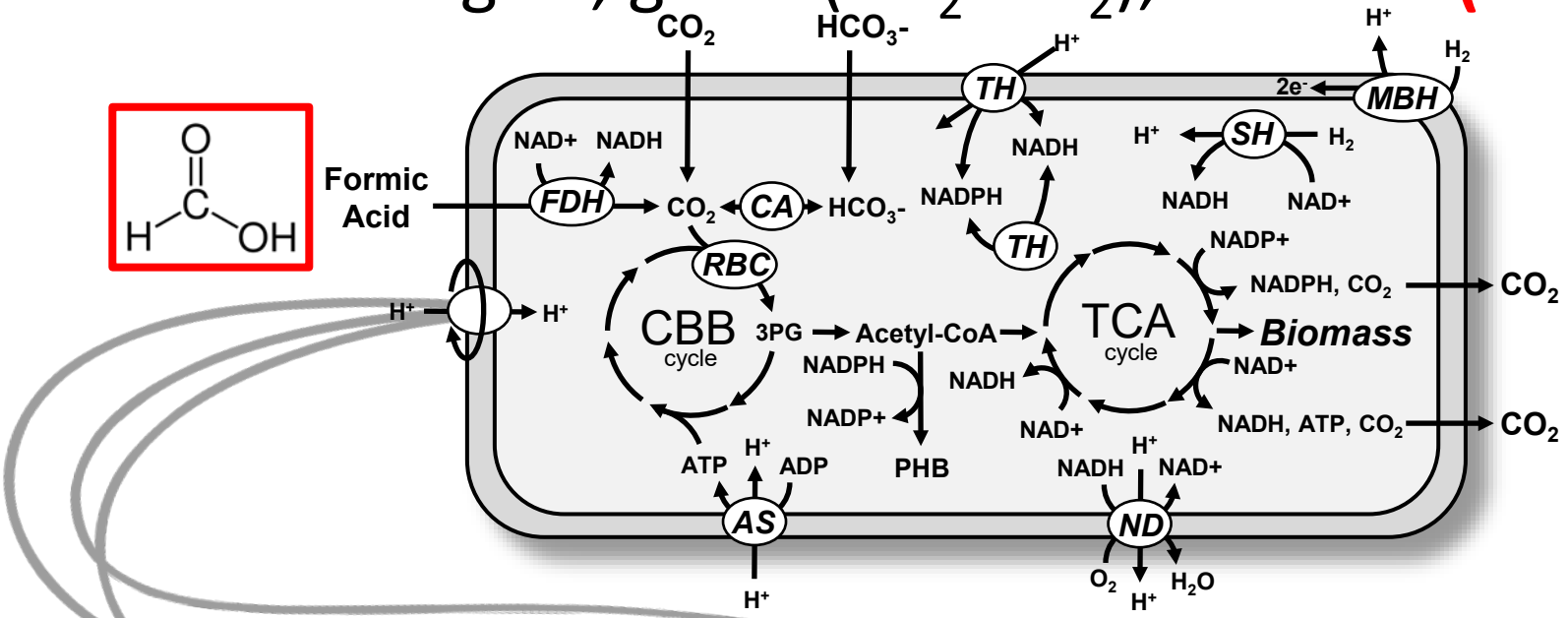
+ 35,000,000,000 mt/year CO_2

Southern Hemisphere Sea Ice Extent, climatereanalyzer.org

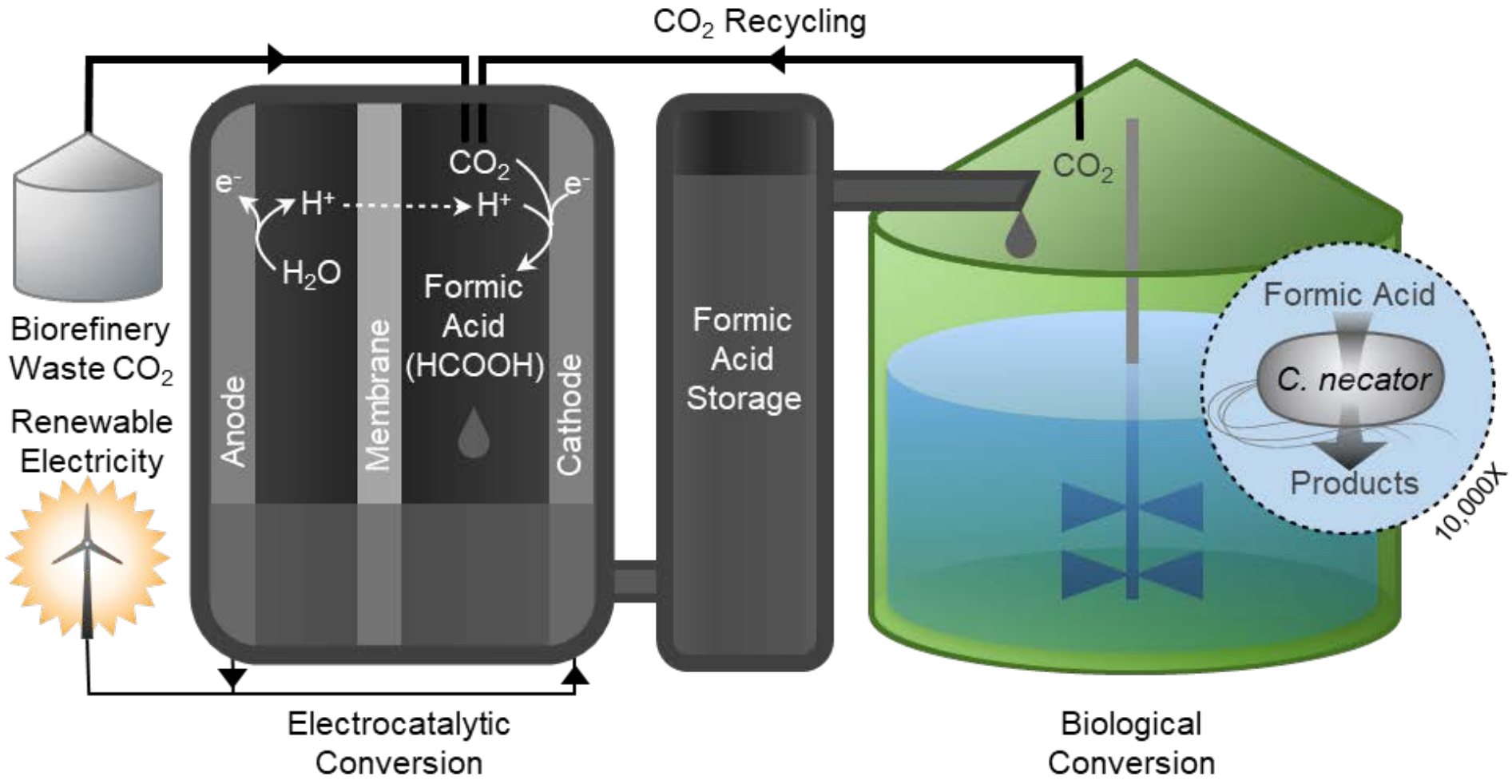


Introduction to *Cupriavidus necator*

- Well studied Gram-negative bacteria
  - Genome: C1≈ 4 Mbp, C2 ≈ 2.9 Mbp, pHG1 ≈ 0.45 Mbp
- Proven industrial host (PHA bioplastic)
- Very diverse metabolism
  - Growth on sugars, gases (CO_2 + H_2), **formate (CH_2O_2)**



The Dream: A Formate Bioeconomy



The Dream: A Formate Bioeconomy

ACS Energy LETTERS

http://pubs.acs.org/journal/aelccp

A Robust, Scalable Platform for the Electrochemical Conversion of CO_2 to Formate: Identifying Pathways to Higher Energy Efficiencies

Yingying Chen, Ashlee Vise, W. Ellis Klein, Firat C. Cetinbas, Deborah J. Myers, Wilson A. Smith, Todd G. Deutsch, and K. C. Neyerlin*

Cite This: *ACS Energy Lett.* 2020, 5, 1825−1833 Read Online

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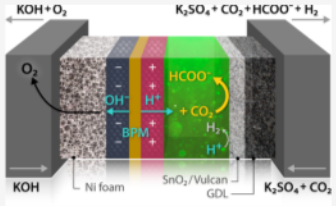
ABSTRACT: This work demonstrated a robust, scalable cell architecture for electroreduction of CO_2 (CO_2R). An up to 90% faradaic efficiency for the conversion of CO_2R to formate at 500 mA/cm^2 was realized at a 25 cm^2 gas diffusion electrode (GDE) with a carbon-supported SnO_2 electrocatalyst. A 1.27 mm thick catholyte was used between the bipolar membrane and cathode GDE, which could be further reduced to tens of micrometers upon refinement. The deconvolution of the potential drop from each individual component/process guides the pathways to higher energy efficiencies of CO_2R at this platform. Significant changes in the agglomerate size and aspect ratio on the electrode before and after an 11 h test were revealed by nano-CT, suggesting reduced CO_2 accessibility from electrode degradation. The versatility of this CO_2R testing platform enables the ability to assess materials, components, and interactions at scales more in line with future devices.



In recent years, remarkable efforts have been dedicated to the electrochemical reduction of CO_2 (CO_2R) into value-added chemicals and fuels, such as carbon monoxide,[1−6] formic acid/formate,[7−12] ethylene,[13−15] ethanol,[14,16−19] methane,[20−23] and methanol.[15,24−27] When coupled with renewable energy sources, CO_2R is an attractive approach for utilizing carbon chemical feed stocks while reducing CO_2 emissions and closing the anthropogenic carbon loop.[28]

Compared to the other CO_2R products, formic acid/formate stands out as one of the few economically viable products due to its high product value per electron.[29] The end-of-life (20 years) net present value of formic acid was calculated to be $39.4 million in a generalized CO_2 electrolyzer system for the temperature.[34−36] Furthermore, formic acid can be used as an effective "in situ" hydrogen source for the conversion of biomass and biomass-derived platform molecules into value-added chemicals.[37] Formate can also be utilized downstream in biological processes using formatotrophs, such as *Cupriavidus necator*,[38] which can be engineered to convert formate to higher alcohols.[39] The advantage of coupling biological systems to the electrochemical production of formate is that many enzymes that can convert formate are also tolerant of minor products that may be formed alongside formate, significantly relaxing the need to achieve a 100% FE for a single product, which has challenged the CO_2R community.

nature COMMUNICATIONS

ARTICLE

https://doi.org/10.1038/s41467-020-17403-1 OPEN

Electrochemical CO_2 reduction to high-concentration pure formic acid solutions in an all-solid-state reactor

Lei Fan[1,2,5], Chuan Xia[1,3,5], Peng Zhu[1], Yingying Lu[2]✉ & Haotian Wang[1,4]✉

Electrochemical CO_2 reduction reaction (CO_2RR) to liquid fuels is currently challenged by low product concentrations, as well as their mixture with traditional liquid electrolytes, such as $KHCO_3$ solution. Here we report an all-solid-state electrochemical CO_2RR system for continuous generation of high-purity and high-concentration formic acid vapors and solutions. The cathode and anode were separated by a porous solid electrolyte (PSE) layer, where electrochemically generated formate and proton were recombined to form molecular formic acid. The generated formic acid can be efficiently removed in the form of vapors via inert gas stream flowing through the PSE layer. Coupling with a high activity (formate partial current densities ~450 mA cm^{-2}), selectivity (maximal Faradaic efficiency ~97%), and stability (100 hours) grain boundary-enriched bismuth catalyst, we demonstrated ultra-high concentrations of pure formic acid solutions (up to nearly 100 wt.%) condensed from generated vapors via flexible tuning of the carrier gas stream.

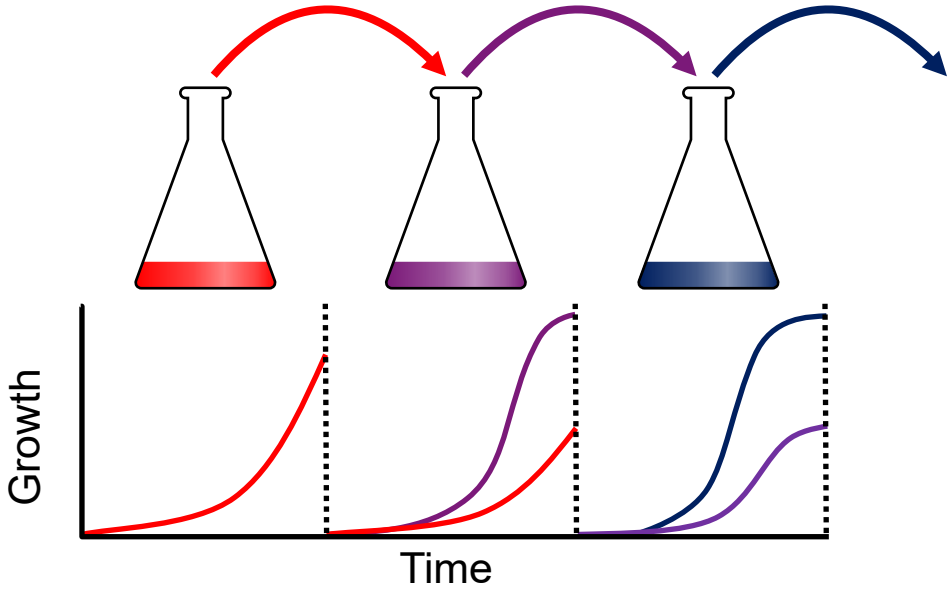
90% faradaic efficiency
Current density: 500 mA/cm^2
87.4 mM formate at 40 mL/min

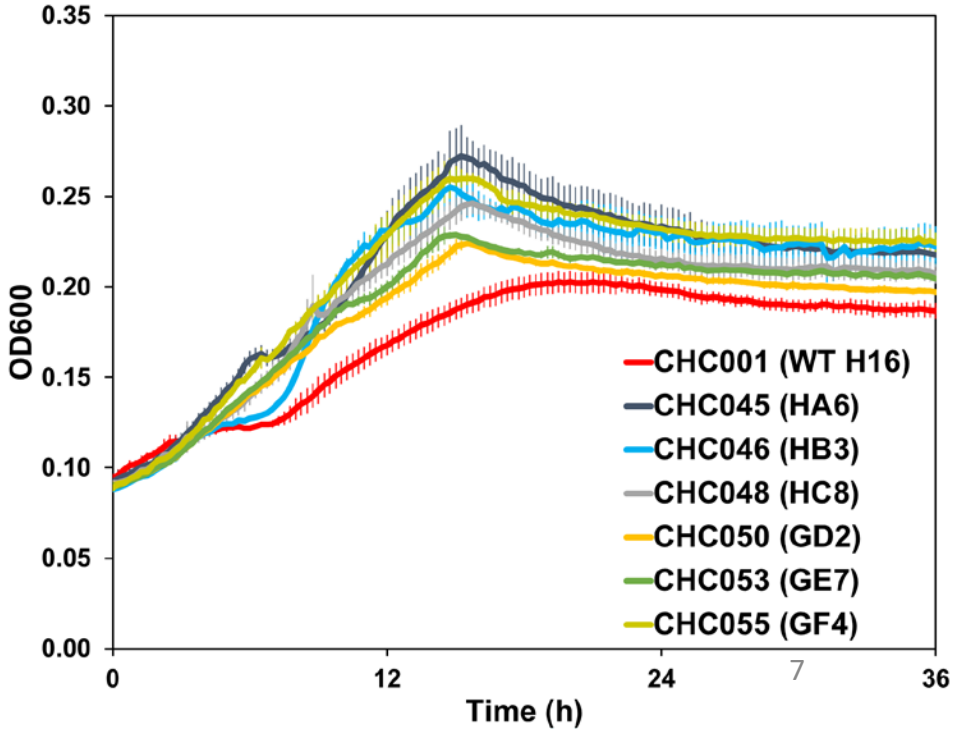
97% faradaic efficiency
Current density: 450 mA/cm^2
100 wt. % formic acid

Adaptive Laboratory Evolution

Improving growth on formate by ALE:

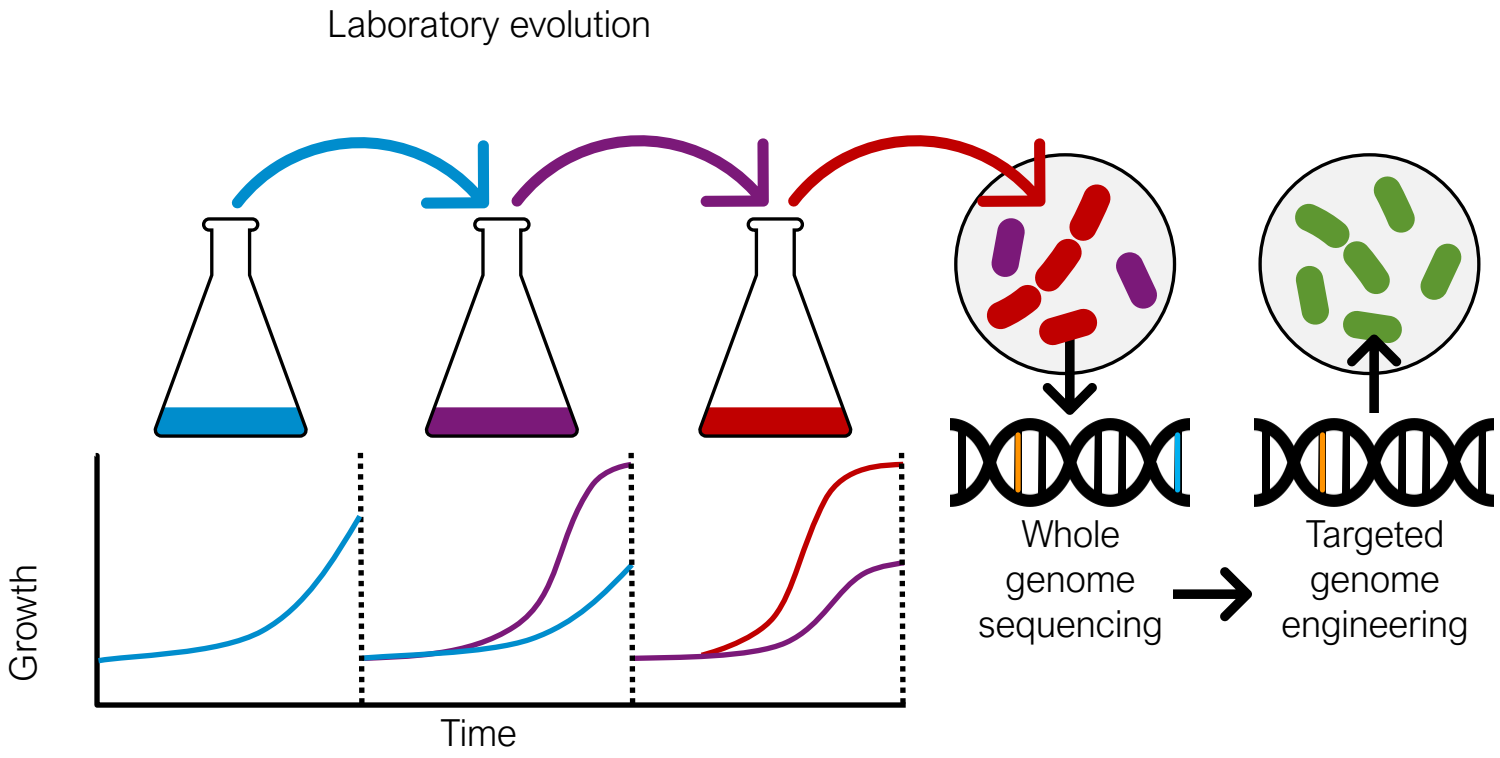
- Six replicate wildtype cultures
- Minimal media + 50mM sodium formate
- Growth for ≈ six months, 400+ generations





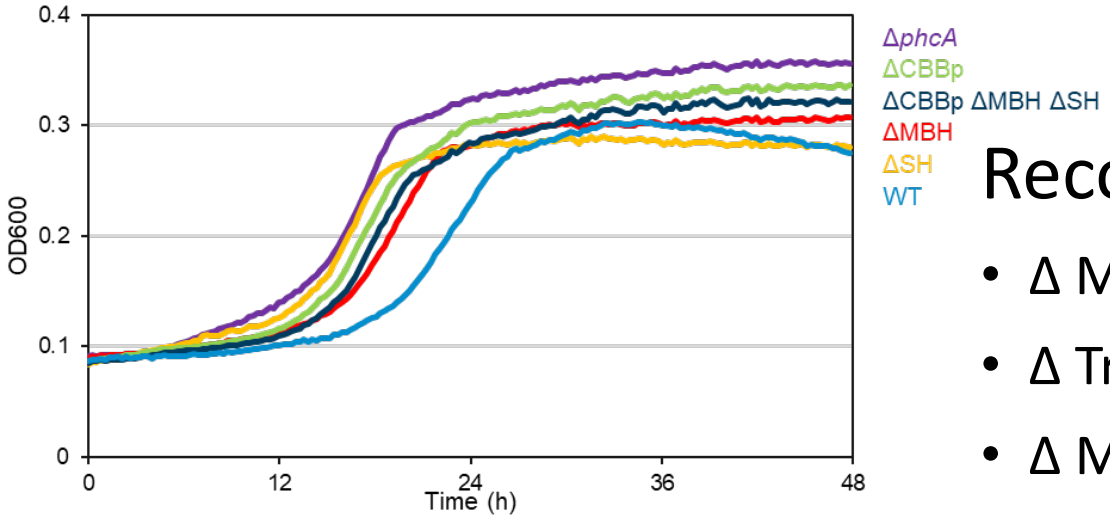
Improving growth on formate by ALE:

- Isolate best evolved strains
- Sequence their genomes
- Recreate by rational engineering



Genome sequencing of ALE strains

	Megaplasmid pHG1				Chromosome 1
	Hydrogenase (membrane)	Hydrogenase (soluble)	CBB Operon	Total Deletion (≈bp)	phcA Regulator
HA6				0	SNP
HB3	Δ		Δ	42,177	SNP
HC8	Δ	Δ	Δ	124,302	
GD2	Δ	Δ	Δ	120,753	SNP
GE7	Δ	Δ	Δ	120,730	SNP
GF4	SNP		Δ	12,282	



Reconstituted ALE strains:

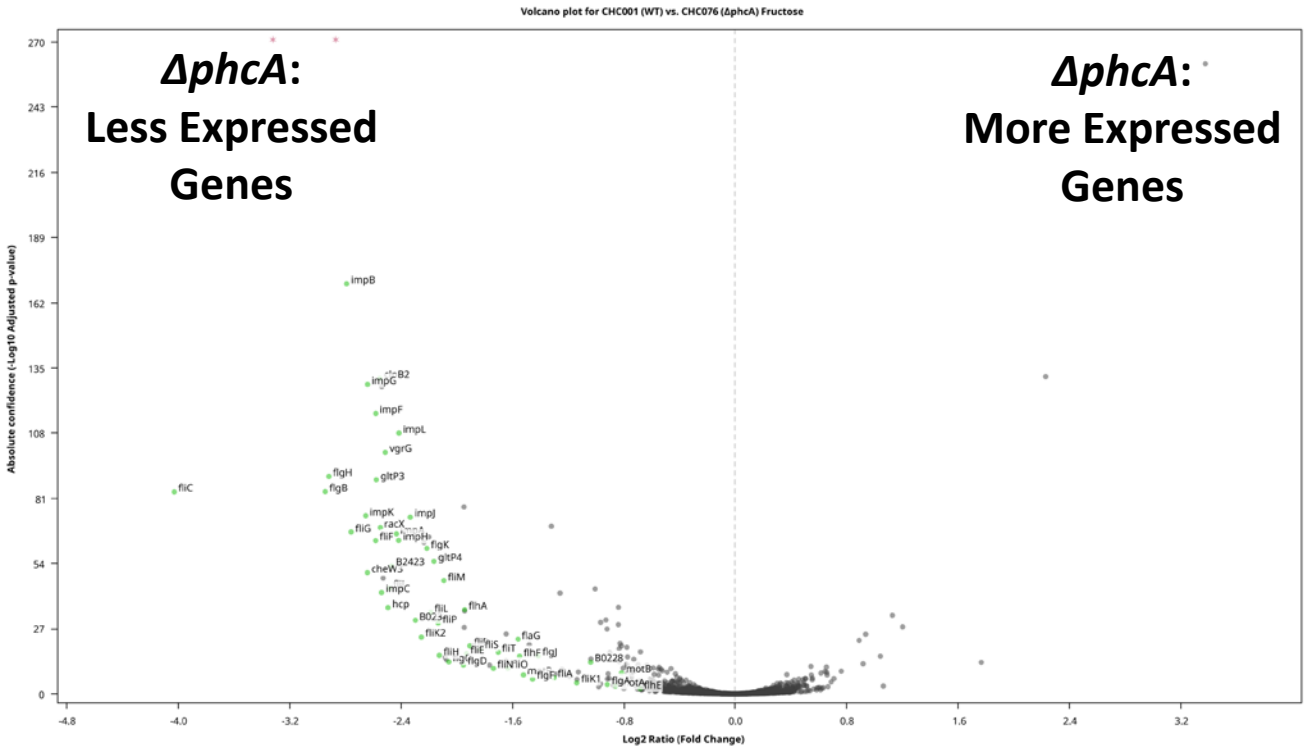
- Δ Megaplasmid CBB Operon (CBBp)
- Δ Transcriptional Regulator (*phcA)*
- Δ Membrane Hydrogenase (MBH)
- Δ Soluble Hydrogenase (SH)

C. necator ALE: Δ*phcA*

	Chromosome 1
	phcA Regulator
HA6	SNP
HB3	SNP
HC8	
GD2	SNP
GE7	SNP
GF4	

- Most ALE strains have *phcA* deletions.
- CHC076 (Δ*phcA*): improved growth on formate
- *phcA* = LysR family transcriptional regulator
  - What does it do?

C. necator ALE: Δ*phcA* RNA Seq

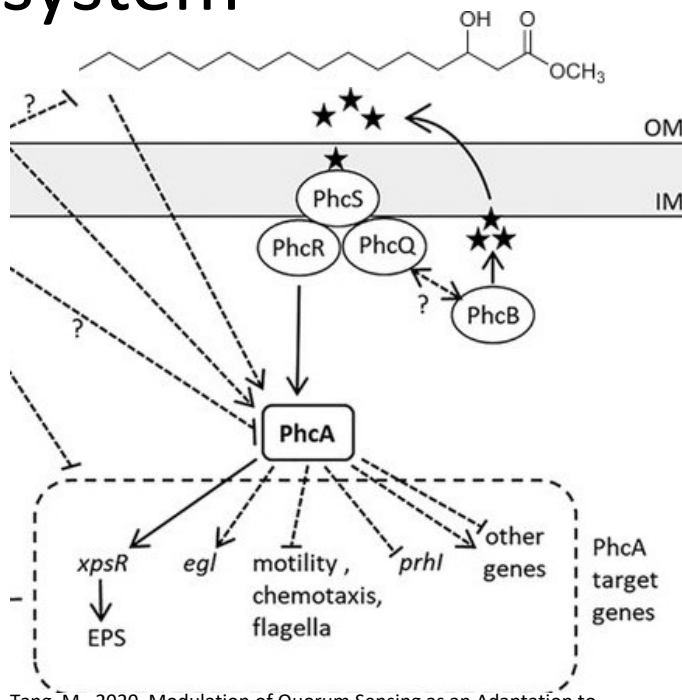


- CHC001 (WT) vs. CHC076 (Δ*phcA*) [Fructose/Formate]
- PhcA controls: flagella, chemotaxis, adhesion, secretion
  - Activates expression of 100's of genes

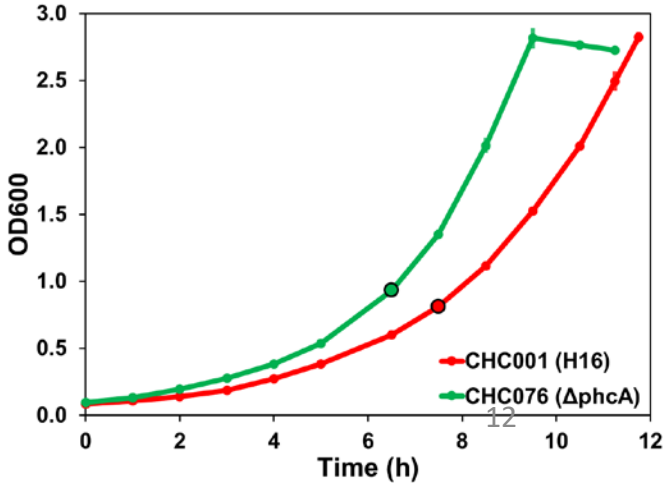
(H16_B2360-B2373)	fliC	flaG	fliD	fliS	fliT	fliK1	flhB2	fliE	fliF	fliG	fliH	fliI	fliJ	fliK2
CHC001(WT) Formate	805.2	40.1	45.1	14.3	20.7	1.4	0.4	0.5	0.9	1.2	0.3	0.9	1.2	0.3
CHC001 (WT) Fructose	348.1	40.9	35.2	11.2	11.9	1.4	0.8	8.1	9.1	15.7	2.6	6.1	13.4	2.7
CHC076 (ΔphcA) Formate	18.6	11.2	7.6	3.0	4.9	0.5	0.3	0.2	0.6	0.4	0.1	0.7	0.7	0.1
CHC076 (ΔphcA) Fructose	11.4	12.6	7.3	2.5	3.0	0.5	0.2	1.5	1.3	1.9	0.3	1.4	1.9	0.4

C. necator ALE: Δ*phcA*

- *R. solanacearum* quorum sensing system
  - PhcA controls virulence factors
    - Only during (high OD) plant invasion
  - Uses signaling molecule 3OH-PAME
- *C. necator*: also quorum sensing!
  - H16 PhcA: very similar regulon
- Deletion of *phcA* during ALE?
  - Reduces unnecessary expression
  - Conserves valuable ATP
- Δ*phcA*: broad utility
  - **Improves growth on fructose!**
  - Improves growth on succinate!



Tang, M., 2020. Modulation of Quorum Sensing as an Adaptation to Nodule Cell Infection during Experimental Evolution of Legume Symbionts



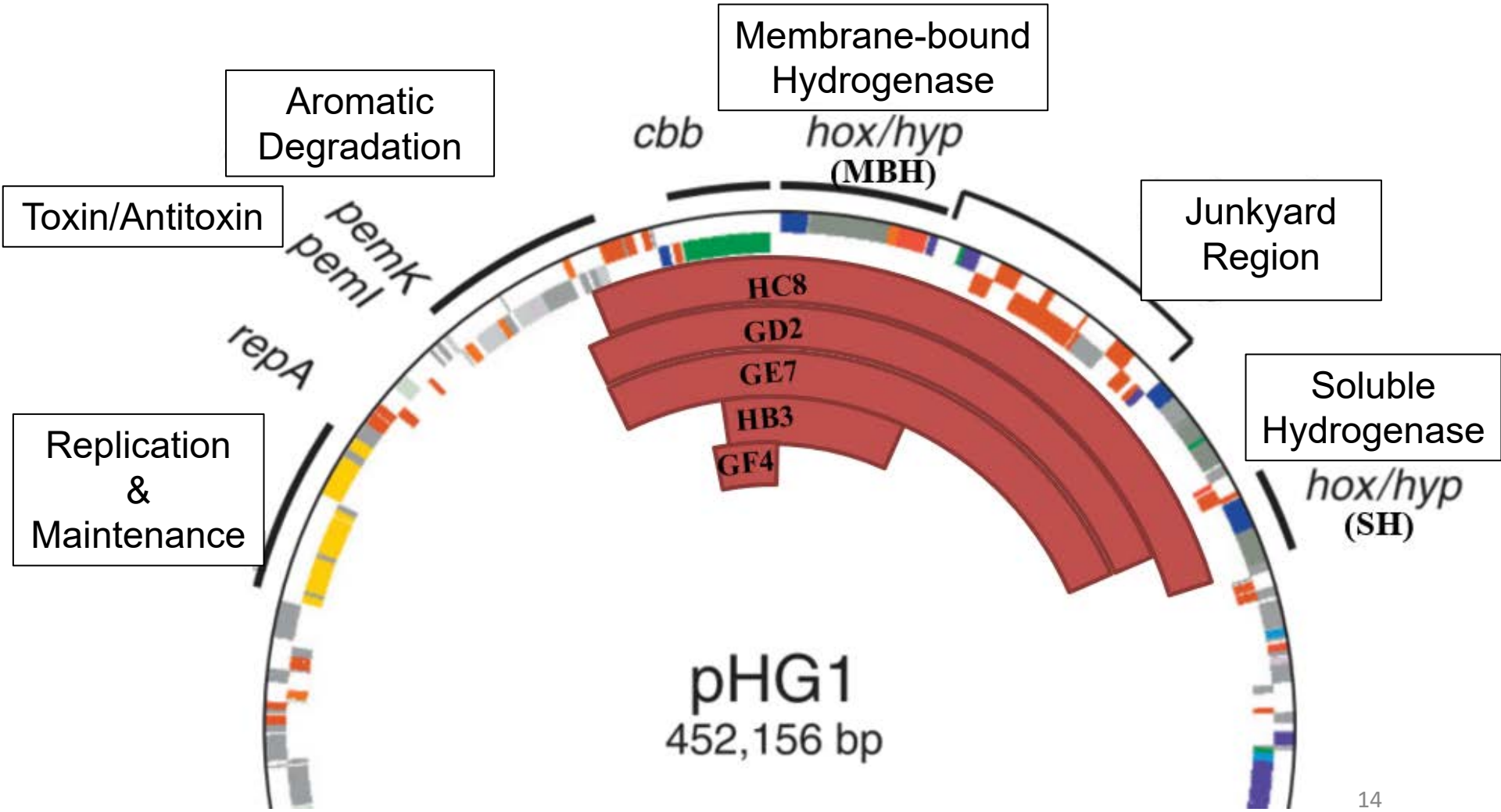
C. necator ALE: Megaplasmid Deletions

	Megaplasmid pHG1			
	Hydrogenase (membrane)	Hydrogenase (soluble)	CBB Operon	Total Deletion (≈bp)
HA6				0
HB3	Δ		Δ	42,177
HC8	Δ	Δ	Δ	»124,302«
GD2	Δ	Δ	Δ	»120,753«
GE7	Δ	Δ	Δ	»120,730«
GF4	SNP		Δ	»12,282«

- Most ALE strains have deletions in pHG1.
  - CHC077: ΔMBH
  - CHC078: ΔSH
  - CHC079: ΔCBBp
- All show improved growth on formate, but why?

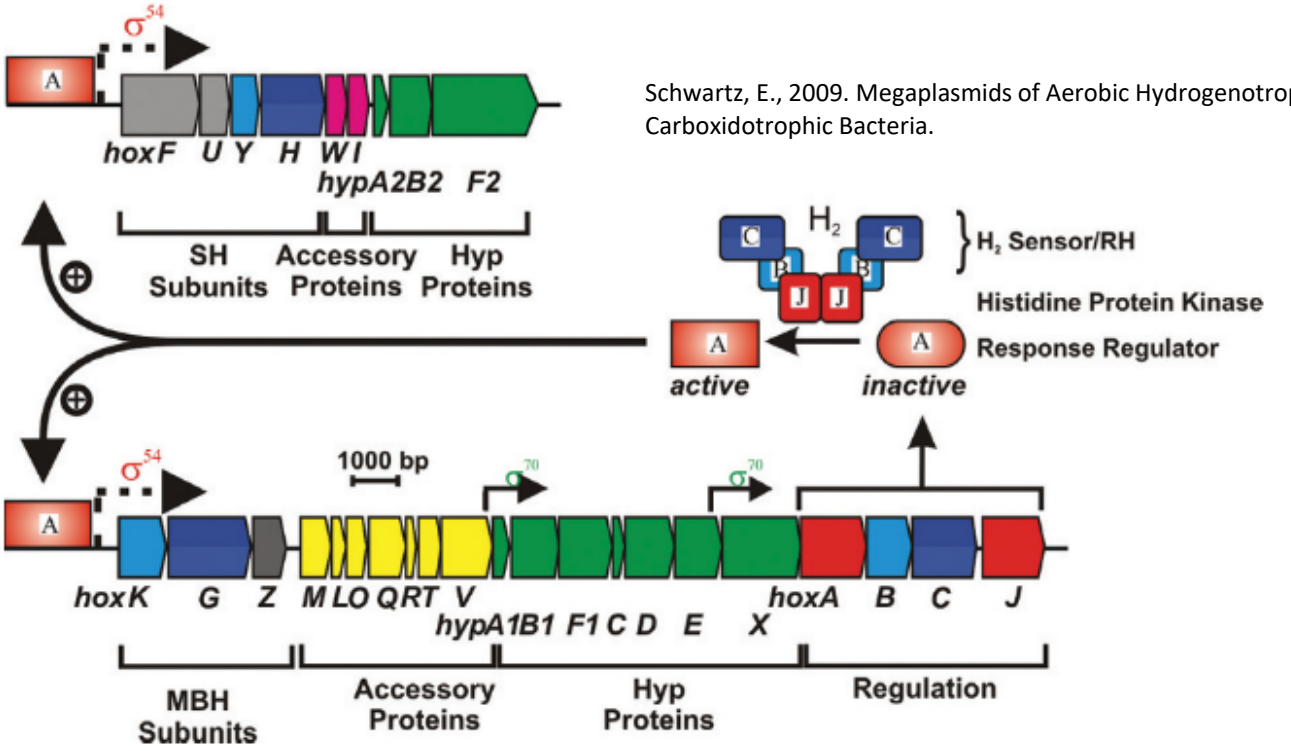
Genome sequencing of ALE strains

Huge deletions found in the megaplasmid pHG1



Mergeay, M. 2009. Megaplasmids in *Cupriavidus* Genus and Metal Resistance

C. necator ALE: Δ Hydrogenases

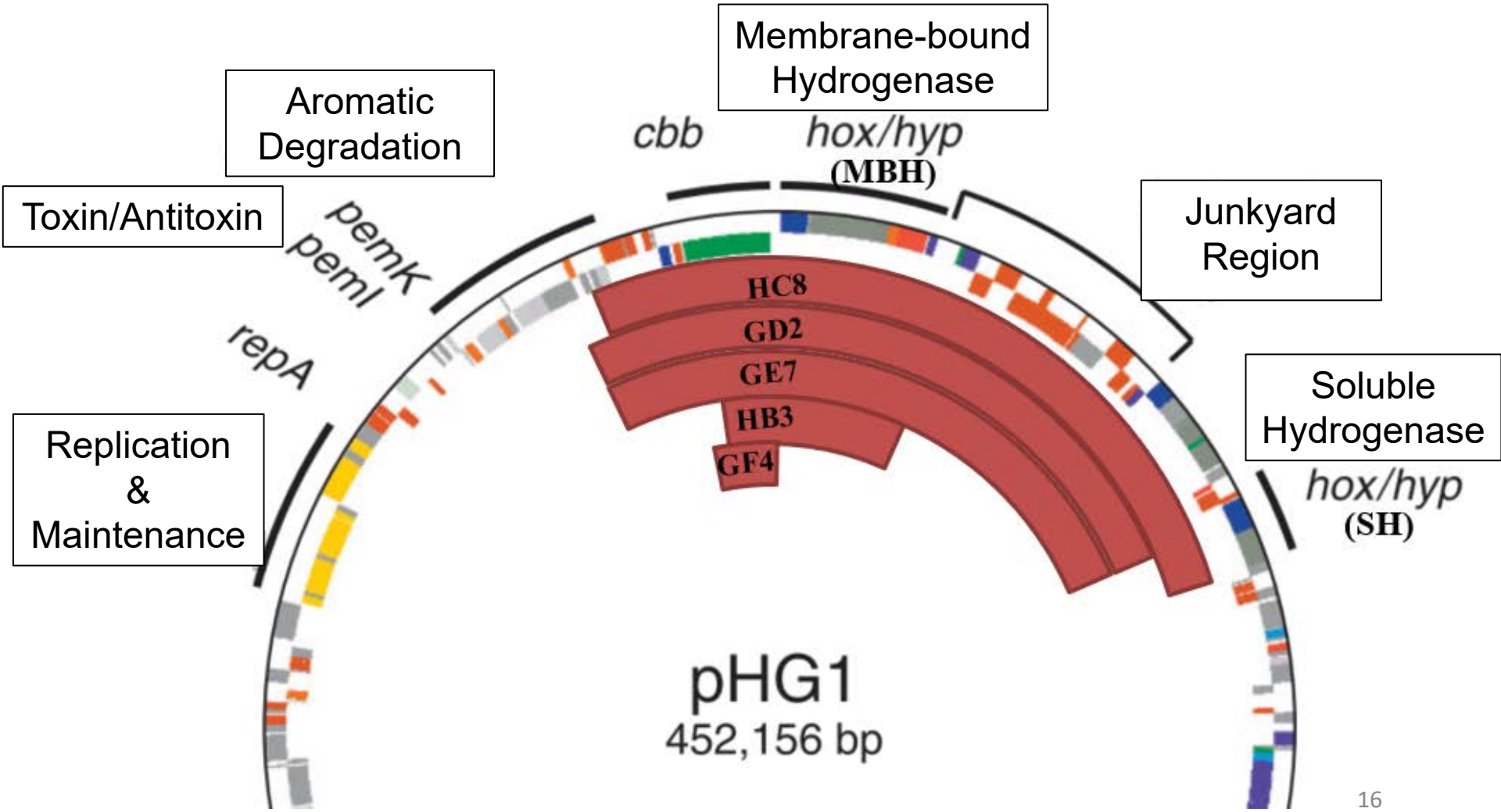


Schwartz, E., 2009. Megaplasmids of Aerobic Hydrogenotrophic and Carboxidotrophic Bacteria.

- Hydrogenases are expressed even when not needed
- Hydrogenase production is energetically expensive
  - Account for up to 3% of the proteome by mass!
- Deletion of SH/MBH during formate ALE?
  - Conserves valuable carbon and energy!

Genome sequencing of ALE strains

Huge deletions found in the megaplasmid pHG1

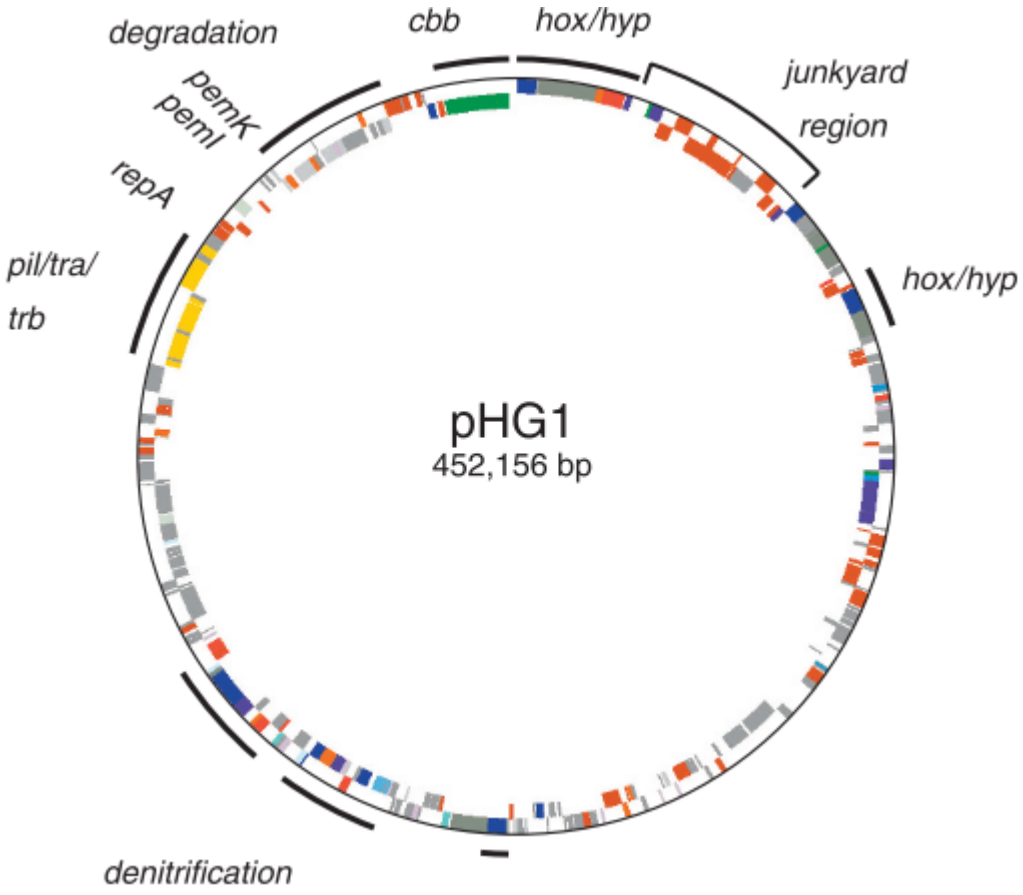


Mergeay, M. 2009. Megaplasmids in *Cupriavidus* Genus and Metal Resistance

C. necator ALE: Δ Megaplasmid pHG1

Plasmid addiction system:

- ***pemK***: Toxin
  - mRNA endoribonuclease
  - Stable
- ***pemI***: Antitoxin
  - PemK inhibitor
  - Unstable
- Ensures pHG1 inheritance

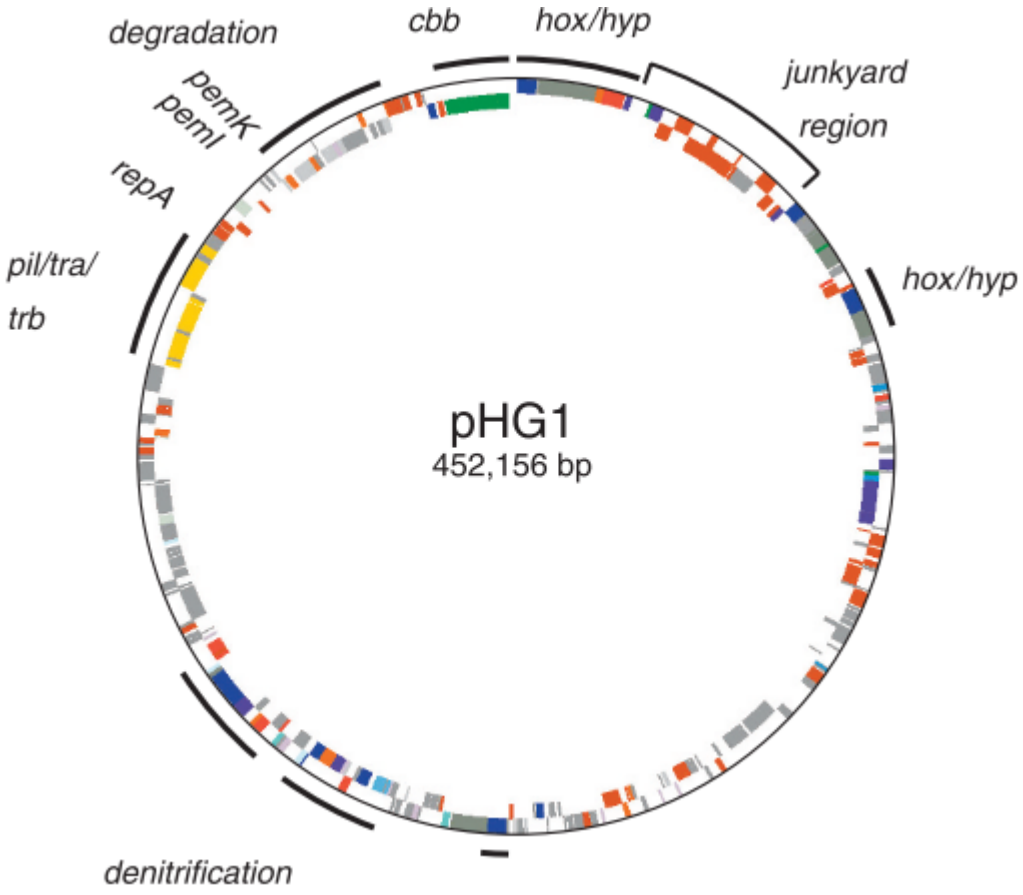


- Step 1: pCHC027 (Δ*pemK*)
- Removes megaplasmid addiction system

C. necator ALE: Δ Megaplasmid pHG1

pHG1 Replication Region

- Origin of replication:
  - **oriV**
- Replication initiation:
  - ***repAB***
- Plasmid partitioning:
  - ***parAB***
- Helicase:
  - ***helD***



- Step 2: pCHC036 (Δrep)
- Removes 9kb replication cluster

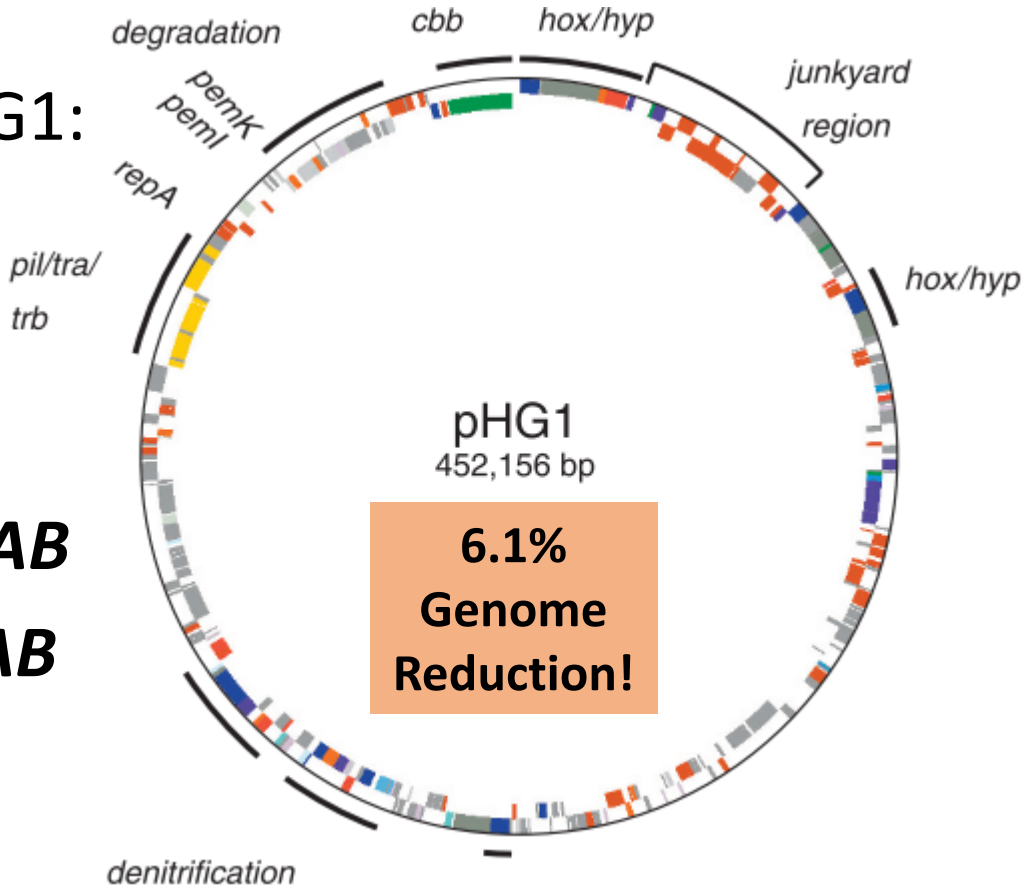
C. necator ALE: Δ Megaplasmid pHG1

Two stage strategy for ΔpHG1:

1) Δ Addiction toxin: ***pemK***

2) Δ Replication region

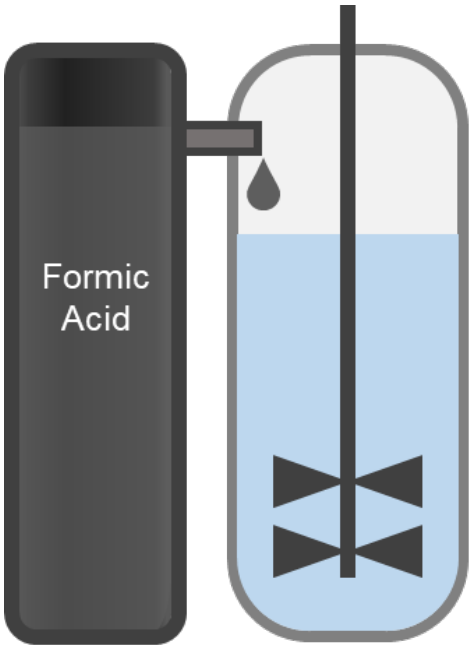
- Origin of replication: **oriV**
- Replication initiation: ***repAB***
- Plasmid partitioning: ***parAB***
- Helicase: ***helD***

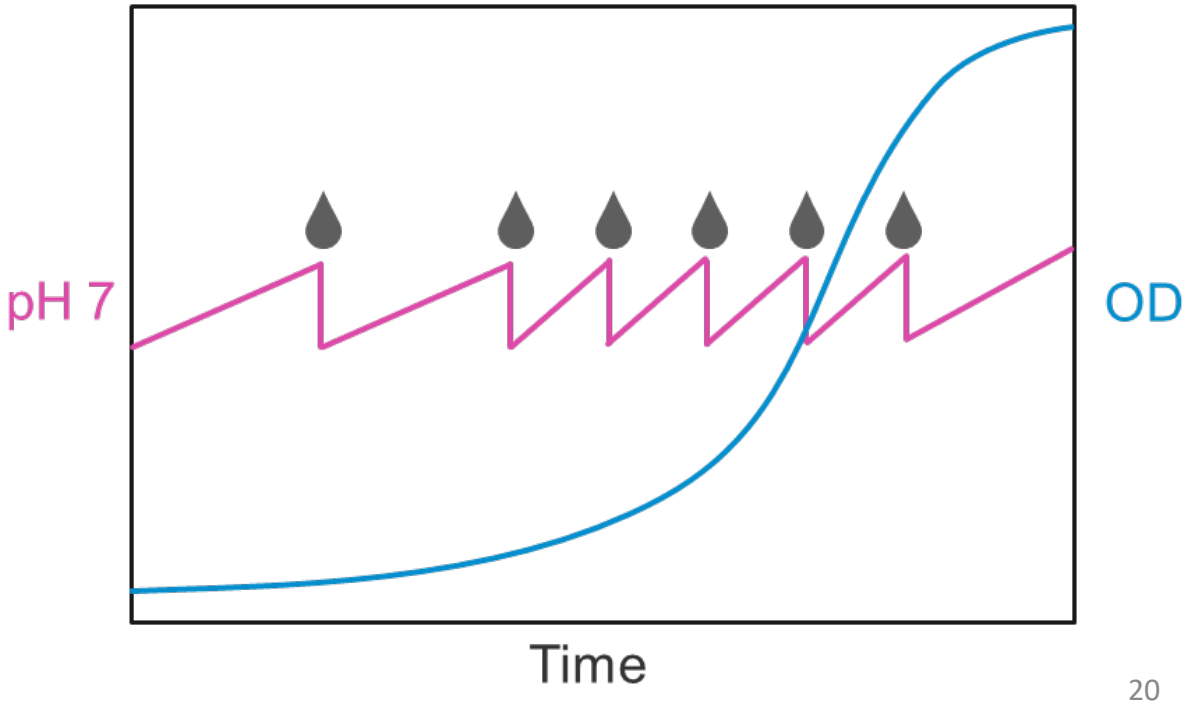


- **Successfully deleted the entire megaplasmid!**
- Strain CHC105 (ΔpHG1)
  - Improved growth on both formate and fructose

pH-stat bioreactor cultivation

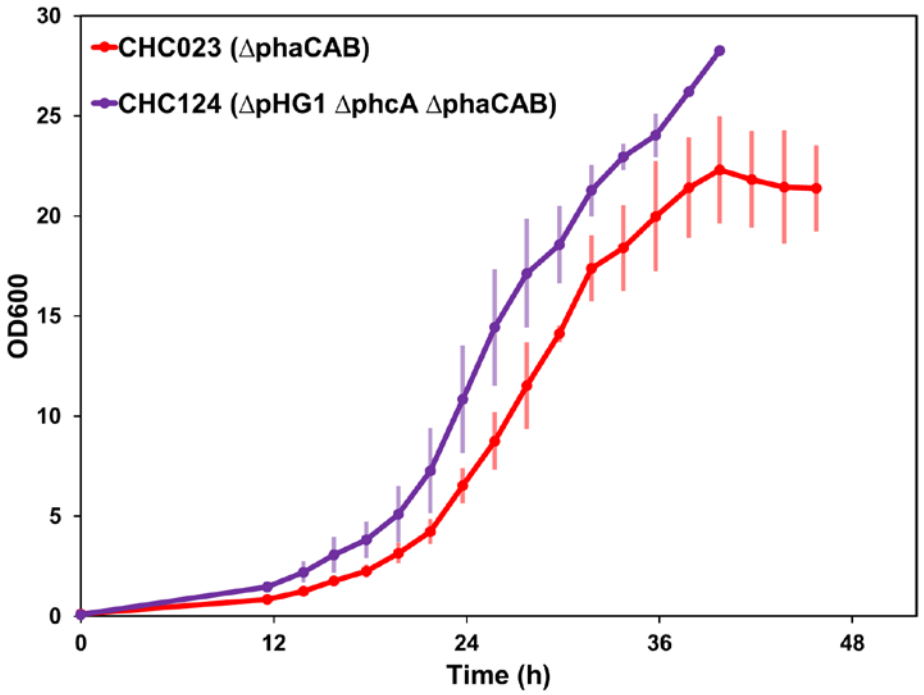
- Formic acid consumption → raises pH
- pH (setpoint 6.8) controlled with a **25% formic acid feed**
- Formic acid is fed exactly as quickly as it is consumed
- Concentration of formic acid in the bioreactor is minimized

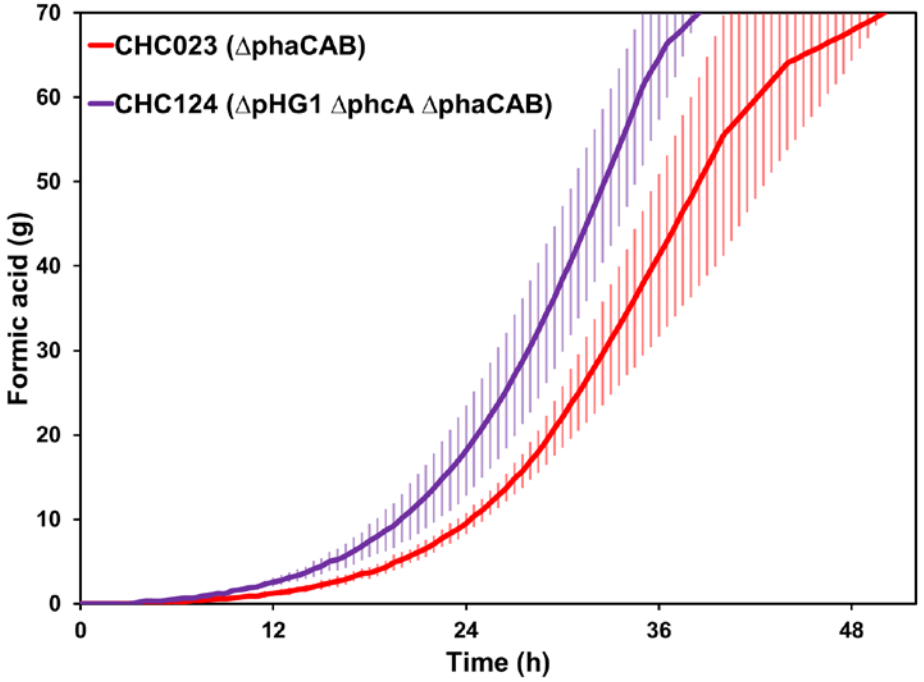




C. necator ALE: Bioreactor Runs

- 500 mL bioreactors
  - 35% formic acid feed
  - pH-stat feeding method
- Best ALE-inspired strains
  - ΔpHG1
  - Δ*phcA*
  - Also Δ*phaCAB*
- Excellent performance:
  - 24% faster growth
  - 32% faster feeding
  - **SOTA for formatotrophy!**





Metabolic Engineering 75 (2023) 78–90

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journal homepage: www.elsevier.com/locate/meteng

Improving growth of *Cupriavidus necator* H16 on formate using adaptive laboratory evolution-informed engineering

Christopher H. Calvey [a], Violeta Sànchez i Nogué [a], Aleena M. White [a], Colin M. Kneucker [a], Sean P. Woodworth [a], Hannah M. Alt [a], Carrie A. Eckert [b], Christopher W. Johnson [a,*]

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Quorum sensing
phcA

ABSTRACT

Conversion of CO_2 to value-added products presents an opportunity to reduce GHG emissions while generating revenue. Formate, which can be generated by the electrochemical reduction of CO_2, has been proposed as a promising intermediate compound for microbial upgrading. Here we present progress towards improving the soil bacterium *Cupriavidus necator* H16, which is capable of growing on formate as its sole source of carbon and energy using the Calvin–Benson–Bassham (CBB) cycle, as a host for formate utilization. Using adaptive laboratory evolution, we generated several isolates that exhibited faster growth rates on formate. The genomes of these isolates were sequenced, and resulting mutations were systematically reintroduced by metabolic engineering, to identify those that improved growth. The metabolic impact of several mutations was investigated further using RNA-seq transcriptomics. We found that deletion of a transcriptional regulator implicated in quorum sensing, PhcA, reduced expression of several operons and led to improved growth on formate. Growth was also improved by deleting large genomic regions present on the extrachromosomal megaplasmid pHG1, particularly two hydrogenase operons and the megaplasmid CBB operon, one of two copies present in the genome. Based on these findings, we generated a rationally engineered Δ*phcA* and megaplasmid-deficient strain that exhibited a 24% faster maximum growth rate on formate. Moreover, this strain achieved a 7% growth rate improvement on succinate and a 19% increase on fructose, demonstrating the broad utility of microbial genome reduction. This strain has the potential to serve as an improved microbial chassis for biological conversion of formate to value-added products.

Perspectives: Engineering for Alternative Feedstocks

- No microbes on earth evolved under your specific, controlled laboratory conditions!
  - We can harness the power of ALE to improve naturally occurring microbes on any feedstock.
- Many microbes are “generalists” – capable of pivoting towards many alternate growth modes.
  - In nature, agility is advantageous
  - In the lab, this is energetically wasteful
- Consider also the concept of “genome reduction.”
  - Much of the genome may be dispensable, and even worse than useless, under your growth conditions!

Team Members:

- PI: Chris Johnson
- Violeta Sànchez i Nogué
- Carrie Eckert
- Aleena White
- Lucas Friedberg
- Colin Kneucker
- Kelsey Ramirez
- Sean Woodworth
- Stephan Haugen
- Hannah Alt
- Michelle Reed

BETO

- Ian Rowe
- Chenlin Li

Thank You!

Q&A

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BIOENERGY TECHNOLOGIES OFFICE

NREL Transforming ENERGY

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