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PROGRESS TOWARDS A NEW MODEL CHEMOLITHOAUTOTROPHIC HOST

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Cooperative Research and Development Agreement (CRADA) Final Report

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In accordance with Requirements set forth in the terms of the CRADA, this document is the CRADA Final Report, including a list of Subject Inventions. It is to be forwarded to the DOE Office of Scientific and Technical Information upon completion or termination of the CRADA, as part of the commitment to the public to demonstrate results of federally funded research.

Parties to the Agreement: Kiverdi Inc, NREL, ORNL and Berkeley Lab

CRADA number: FP00006779

CRADA Title: Progress towards a new model chemolithoautotrophic host

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Sponsoring DOE Program Office(s):

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LBNL Report Number:

[PI to complete]

OSTI Number:

[SPO to complete]

Joint Work Statement Funding Table showing DOE funding commitment:

DOE Funding to LBNL	\$347,200
DOE Funding to NREL	\$308,600
DOE Funding to ORNL	\$244,200
Participant Funding to LBNL	0
Participant In-Kind Contribution Value	\$385,700
Total of all Contributions	\$1,285,700

Provide a list of publications, conference papers, or other public releases of results, developed under this CRADA:

None

Provide a detailed list of all subject inventions, to include patent applications, copyrights, and trademarks:

(Patents and patent applications are to include the title and inventor(s) names. When copyright is asserted, the Government license should be included on the cover page of the Final Report)

None

Executive Summary of CRADA Work:

Some of the largest scale chemical processes for the production of important commodity chemicals and fuels involve an H₂-containing gas mixture as an intermediate [e.g. ammonia, urea, methanol, Fischer-Tropsch (F-T) diesel, and hydrotreatment of crude petroleum to refined fuels]. The successful development of a model H₂-oxidizing chemoautotrophic host could expand the range of fuels and chemicals produced from H₂ and CO₂ intermediates in the chemical, oil, and gas sectors in the near term, as well as from renewable and waste-derived sources of these gases that are expected to greatly expand in coming years. Among non-photosynthetic bacteria that can utilize H₂ and CO₂, *Cupriavidus necator* (*C. necator*, formerly *Ralstonia eutropha*), is the best studied. *C. necator* is an excellent microbial host for the production of a variety of chemicals because it grows extremely quickly to very high cell densities autotrophically on H₂ and CO₂, is genetically tractable, and has the ability to accumulate polymers, such as polyhydroxybutyrate, at industrial levels.

Despite having great potential as a platform bioproduction host, genetic tools are limited, making metabolic engineering of this organism slow and laborious. In this CRADA project, we developed a number of genetic tools for *C. necator*:

- 1) Improvement of *C. necator* genetic transformation efficiency
- 2) Integration of heterologous genes into the *C. necator* chromosome and

development of promoter library

3) Development of graded RBSs to control heterologous protein expression

4) Use of RBSs to demonstrate fatty alcohol production in *C. necator*

5) Demonstration of CRISPR-Cas9 gene editing in *C. necator*

Summary of Research Results:

Summary of Research Results: the technical elements were divided into five separate research tasks. The results from each task will be summarized below:

Task 1. Method(s) for 10-fold improved transformation efficiency in *C. necator* (ORNL)

To improve transformation efficiency of WT *C. necator* H16, we deleted a native restriction system in *C. necator* H16 that was recently shown to improve transformation efficiency by approximately 2000-fold (Biotechnol Biofuels 2018 Jun 20;11:172). We recreated this gene deletion and tested a new electro-competent cell preparation protocol using room-temperature stationary phase cells. This shift dramatically decreases prep time, making the new protocol/strain significantly more facile to work with, while simultaneously increasing transformation efficiency by approximately an additional 100-fold over the recently published values.

Phage integrases facilitate site-specific recombination between two specific short sequences of DNA called *attB* and *attP* sites. We integrated *attB* recognition sites for 10 different phage integrases into the chromosome of *C. necator* H16. Into this strain we transformed a suicide plasmid harboring the corresponding integrase recognition site. We observed site specific

recombination between the integrated site and the site on the suicide vector. The integration was mediated by transient expression of the phage integrase on another suicide vector. We identified several phage integrases that demonstrated high transformation efficiency ($>10^5$ cfu/ μ g DNA) (Figure 1A) and greater than 90% specificity for integration at the correct recombination site (Figure 1B), including BXB1, TG1, R4, and BL3. This achievement represents a radical increase in efficiency for chromosomal insertion of heterologous genes and pathways.

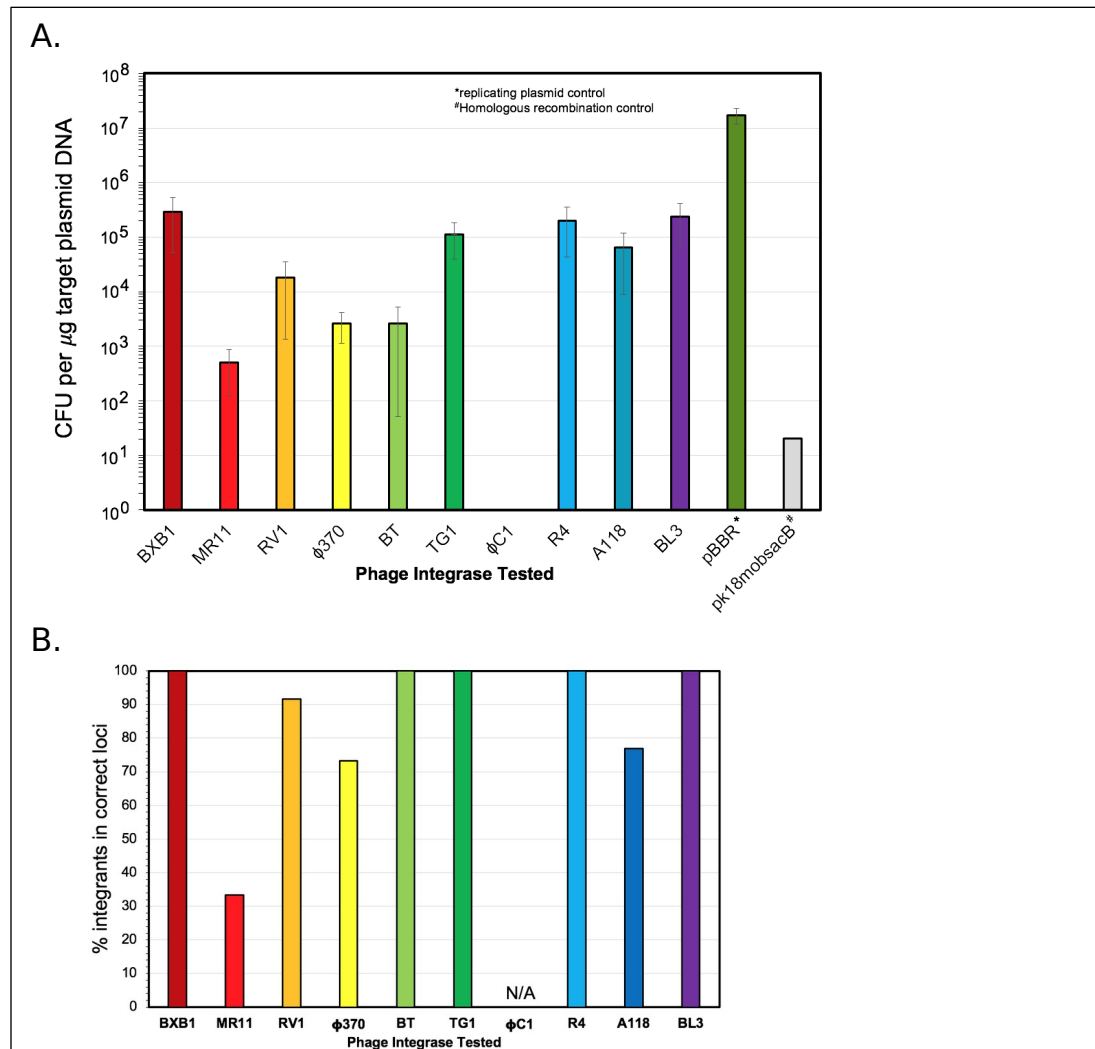


Figure 1. Efficiency of vector integration via phage integrase-mediated site-specific recombination. A) Transformation efficiency of integrating vectors into phage integrase sites and controls (homologous recombination control pK18mobsacB and replicating plasmid control pBBR). B) Specificity of insertion, as the percentage of transformants that had correctly inserted

We utilized the phage integration system to measure the expression of a variety of promoters that were chromosomally integrated. This is a step forward from the current state of the art promoter libraries which are all plasmid-based. We integrated a library of promoters expressing a reporter gene mKate2 (Figure 2A); we then measured RFU/OD600 for each promoter. From the library we observed a 440-fold range of expression (Figure 2B).

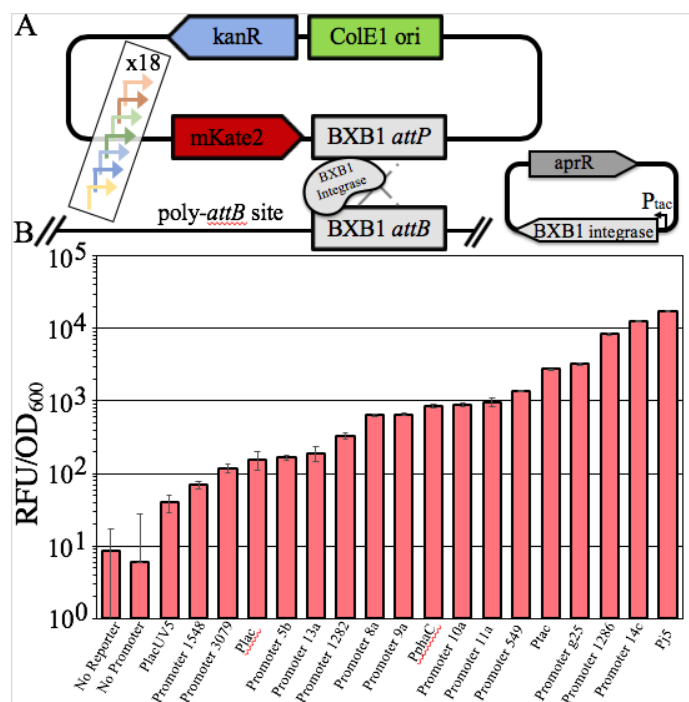


Figure 2. Promoter library expression. A) Diagram of transformation with phage integrase system; B) Relative expression of each promoter.

Although constitutive promoters are useful, often inducible expression of a gene of interest is required for effective biocatalyst development. To this end, we built a T7 RNA polymerase (T7RNAP)-based system for nitrogen starvation-induced expression. We built three strains: QP1000 (WT *C. necator* with phage integrase landing sites), QP1001 (QP1000 with replacement of

nitrogen inducible gene *phaZ2* with T7RNAP), and QP1002 (QP1000 with replacement of nitrogen inducible gene *phaZ2* with T7RNAP and constitutive expression of T7 lysozyme LysY). Into each strain we integrated 5 different strength T7 promoters driving reporter gene mKate2. We then evaluated the expression of the reporter gene in nitrogen rich and poor stationary phase as well as during log growth (Figure 3 A,C,E). We observed that all strains with T7RNAP showed strong nitrogen induction of the reporter gene, however the strain without LysY had leaky expression in log growth (Figure 3 B,D,F). The strain expressing LysY (QP1002) had much lower background mKate2 expression during log growth than the strain without (QP1001) (Figure 3 E,F).

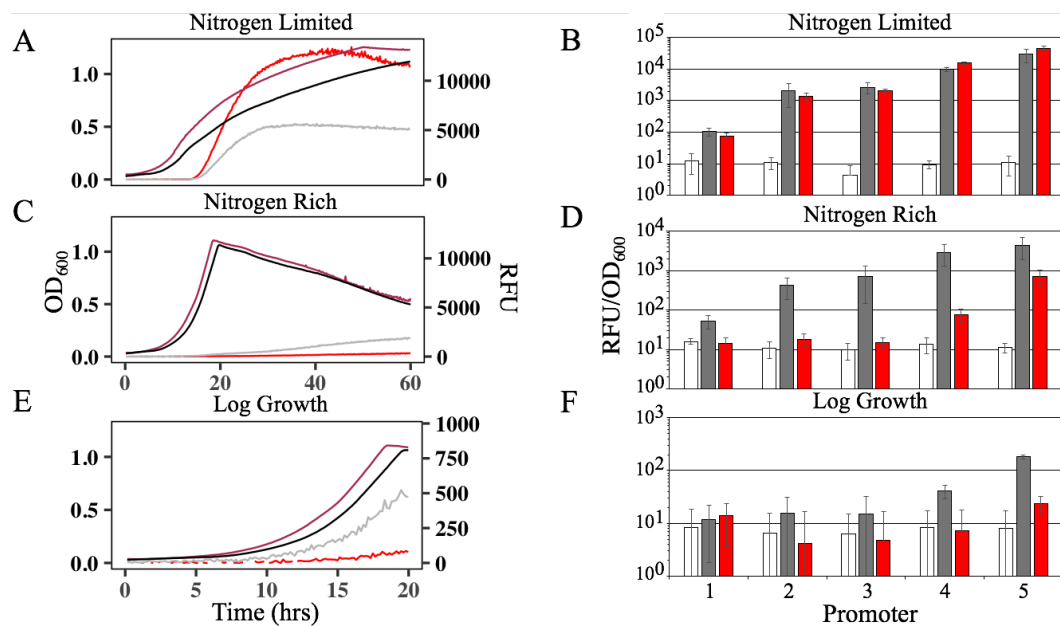


Figure 3: Characterization of T7RNAP activity in *C. necator* under A,B) Nitrogen-limited, C,D) Nitrogen-excess, and E,F) zoomed in on nitrogen-excess exponential growth phase for strains with inserted T7 promoters: QP1000 (white bars in B, D, and F), QP1001 (grayscale lines and bars in all panels), QP1002 (red lines and bars in all panels). A,C,E) The growth (black and maroon lines) and fluorescence (gray and red lines) with the strongest T7 promoter (promoter #5) driving mKate2 expression. B,D,F) Promoter expression levels (RFU/OD₆₀₀) for each strain-promoter pairing are shown.

Task 2: Developing CRISPR/Cas9 editing in *C. necator*

We have designed and cloned three guide RNAs targeting mNEON green that can be used to test both CRISPR cutting and editing as well as CRISPR interference (CRISPRi) in *C. necator* H16 that has mNEON green integrated into the genome. We used the pK18mob suicide plasmid to insert mNEON green into the H16:0006 strain at the *phaCAB* site. Using a plate reader, we tested the fluorescence of this strain and fluorescence increased with growth, indicating successful replacement (also validated by colony PCR). Each gRNA designed was Gibson assembled into the pBTBX5 plasmid separately and correct insertion was confirmed by colony PCR and Sanger sequencing. We conjugated S17 cells containing either the empty pBTBX5 vector or each of the 3 pBTBX5-gRNA plasmids with an mNEON green integration strain (*attB* pQP314) also carrying a pBTBX2-Cas9 plasmid. Unlike with the control pBTBX5 plasmid, zero colonies were detected on plates with the pBTBX5-gRNA plasmids, indicating cell death by each guide RNA and thus an active CRISPR system for use in *C. necator*. A homology directed repair template has also been designed containing a stop codon to disrupt mNeon green, allowing us to measure loss of fluorescence and consequently editing efficiency of the CRISPR system. We have additionally inserted dCas9 into the *attB* pQP314 strain which we then conjugate each gRNA into as well to validate CRISPR interference in the strain as well demonstrated by a decrease in fluorescence of colonies. We have additionally designed guide RNAs targeting the *phaCAB* operon and *pyrF* (required for growth in the absence of uracil, disruption can be selected for on 5-FOA) to further validate the tools at other loci.

Task 3: Develop RBS library for *C. necator*

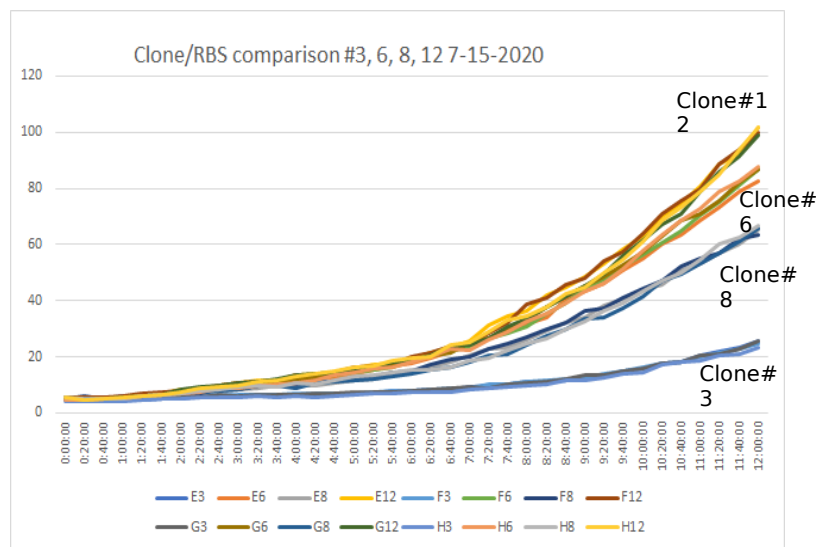
An RBS calculator was used to design a set of RBS of potentially different potencies. A library of 14 independent RBS constructs designed using the Salis RBS Calculator program

(https://salislab.net/software/predict_rbs_calculator) were cloned to create a series of graded expression clones for red fluorescent protein (RFP) differing only in the 20 bases of the ribosome binding domain. All of the plasmids contained a selectable kanamycin gene, a low-copy broad host range origin of replication and AraC promoter so that they could be hosted in both *E. coli* and *Cupriavidus necator*. Plasmids were introduced into *E. coli* and DNA was isolated for sequence determination. In addition, RFP fluorescence was studied in this host using the 96-well plates and a fluorescence reader to demonstrate functionality of the genetic elements under inducing conditions.

Plasmid DNAs were then introduced into *Cupriavidus necator* by electroporation. Overlapping sets of 12 clones were studied in 96-well format on consecutive days with and without arabinose inducer. Extremely consistent fluorescence results were seen among the independent variants (N=4) on consecutive days (Figure 4). In addition, the ranking of strength of the different RBS clones was largely consistent between species. RBS variants could be obtained with low, intermediate and high strengths, suitable for future cloning efforts.

The overall level of expression of all the constructs in *C. necator* was approximately tenfold lower than in *E. coli*. This could reflect many factors such as the codon optimization of the RFP gene constructs which is set up for *E. coli* and not *C. necator*. Selection of kanamycin plasmids in *C. necator* requires much higher levels of the antibiotic than in *E. coli*. This may strongly influence overall expression on the plasmid since the genes for RFP and kanamycin resistance are convergently transcribed and overlap of the transcripts could potentially interfere.

Figure 4.



RFP

fluorescence of *C. necator* strains with graded RBS from Salas RBS calculator. X-axis is time (12 h total) and y-axis is RFP fluorescence.

Task 4: Heterotrophic fatty alcohol production

A library of plasmids containing *tesA* from *E. coli* under different ribosome binding sites preceded by a fatty acyl-CoA reductase (*far*) gene, Maqu2220, under another set of RBSs were constructed and transformed into *C. necator*. These strains were able to produce fatty alcohols up to a titer of > 1 g/L, when cultured in minimal media with 1% fructose. These fatty alcohols were composed primarily of hexadecanol but also other fatty alcohols with

octadecanol being the second most prominent, there were minor levels of dodecanol in the mixture of products.

Task 5. Autotrophic fatty alcohol production

Autotrophic production is currently being performed by Kiverdi.

APPENDIX A (Reference Only)

*This appendix has been developed by DOE to assist DOE Labs in drafting the **Executive Summary** and **Summary of Research Results** sections of the CRADA Final Report.*

Executive Summary of CRADA Work:

Include a discussion of 1) how the research adds to the understanding of the area investigated; 2) the technical -effectiveness of the materials, methods or techniques investigated or demonstrated, and their economic feasibility, if known; and 3) how the project is otherwise of benefit to the public. The discussion should be a minimum of one paragraph and written in terms understandable by an educated layman.

Summary of Research Results:

- *INCLUDE, IF APPLICABLE: "This product contains Protected CRADA Information, which was produced on [DATE] under CRADA No. [##-####] and is not to be further disclosed for a period of [up to and not to exceed] five (5) years from the date it was produced except as expressly provided for in the CRADA."*
- *Summarize project activities for the entire period of performance, including original hypotheses, approaches used, problems encountered, any departure from planned methodology, and an assessment of their impact on the project results. Incorporate technical data, e.g. facts, figures, analyses, and assumptions used during the life of the project to support the technical conclusions of the work. It is acceptable to incorporate the technical data by reference to other publicly available sources, such as a publications or other reports, but not websites. Provide a comparison of the actual accomplishments with the goals and objectives of the project. Where possible, the summary should cover each task listed in the Statement of Work (SOW) and should note any deviations from the project plan, or lack of technical data.*
- *Identify products, potential applications, and technology transfer activities developed under the CRADA, including those completed and anticipated at the time of the report. These include, but are not limited to: 1) networks or collaborations fostered; 2) technologies/techniques/methodologies; 3) other products that reflect the results of the project, such as commercial products, internet sites, data or databases, physical collections, audio or video, software, models, educational aid or curricula, and instruments or equipment.*

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