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Development of an Experimental Pipeline for Onboarding of Actinobacteria with *Rhodococcus ruber* C208 as the Example

by Nathan D Schwalm III, Rebecca L Renberg, Caleb Hellman, Monica Chu, Sean M Halper, and Randi M Pullen

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14. ABSTRACT
Robust microorganisms that tolerate difficult environmental conditions will be required for bioremediation of Army-relevant materials. The development of synthetic biology tools to leverage organisms with these capabilities has lagged behind the development of tools for traditional microbial chassis organisms. Here, we demonstrate the initial experimental steps for development of novel synthetic-biology chassis organisms using *Rhodococcus ruber* C208, a member of the Actinobacteria phylum, as an example. We determine the cultivation conditions, antibiotic sensitivity, transformability, and optimal reporter for use in *R. ruber* C208. Additionally, we sequence the *R. ruber* C208 genome and examine its ability to grow on diverse organic substrates. These experiments provide the necessary first steps to developing more-sophisticated synthetic biology tools for *R. ruber* C208 and establish a pipeline for onboarding of novel synthetic-biology chassis organisms.

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Executive Summary

Synthetic biology could be leveraged to reduce the logistical burden of supporting the Warfighter at the point of need via the remediation of waste materials. The bacterium *Rhodococcus ruber* C208 can degrade polyethylene and other hydrocarbons, but has not yet been developed as a synthetic biology chassis organism. Here, we report the genome sequencing, cultivation, antibiotic sensitivity, and transformability of *R. ruber* C208. Moreover, we characterize the growth of *R. ruber* C208 on several alcohol, aromatic, and dicarboxylate precursor carbon sources, and we determine the optimal fluorescent reporter to use in this strain. These results enable the development of advanced synthetic biology tools for *R. ruber* C208 and provide a proof of concept for the domestication of Actinobacteria species as synthetic biology chassis organisms to support Army missions.

1. Introduction

1.1 Meal, Ready-to-Eat (MRE) Packaging Waste

Generation of plastic waste is a global problem[1] that extends to military-relevant environments. The combined US armed forces generate approximately 14,000 tons of packaging waste from MREs per year.[2] MRE packaging must be highly environmentally resistant to maintain a shelf life of three years at 27 °C or six months at 38 °C.[2] While these traits are beneficial for the stability and transport of MREs to the battlefield, the packaging waste becomes a burden on the Soldier, who must retain it for proper disposal.[3] Targeted remediation of MRE packaging could reduce this logistical burden and potentially provide a useful byproduct. Current MRE packaging consists of an outer low-density polyethylene (LDPE) bag, with interior retort pouches of layered polyethylene, aluminum foil, nylon, and polyester, and interior nonretort pouches of polyethylene, aluminum foil, and polyester.[3] Efforts have been made to modify the composition of MRE packaging to improve its recyclability, but these efforts still require removal of the waste from the field.[2–4]

1.2 *Rhodococcus ruber* Bioremediation

Rhodococcus ruber C208 is a member of the Actinobacteria phylum that was originally isolated as a strain capable of biofilm formation on and limited degradation of polyethylene within 30 days.[5] Subsequent studies identified a copper-binding laccase produced by *R. ruber* C208 that increased polyethylene degradation when overexpressed[6] and the highly hydrophobic nature of *R. ruber* C208 thought to enhance its binding to polyethylene[7] and polystyrene.[8] Other strains of *R. ruber* are known to degrade methoxylated aromatics,[9] polychlorinated bisphenols,[10] phthalates,[11,12] short-chain alkanes (propane, butane, and hexane),[13,14] N-nitrosodimethylamine,[15] and cyclodecanone.[16] The potential that *R. ruber* C208 can degrade polyethylene along with many aromatic carbon sources makes it an attractive synthetic biology chassis to convert aromatic and polymer waste into useful products. Synthetic biology tools exist for the related *Rhodococcus opacus* PD630,[17–19] but tools for genetic manipulation of *R. ruber* strains, outside overexpression[20] and deletion,[21,22] are not currently described.

1.3 Objectives

We sought to enable the use of *R. ruber* C208 as a synthetic biology chassis organism for waste bioremediation. A draft annotated genome sequence of *R. ruber*

C208 was generated. The antibiotic sensitivities of *R. ruber* C208 and growth characteristics in several media were determined to facilitate laboratory cultivation. An electroporation protocol for the transformation of plasmid DNA into *R. ruber* C208 was improved by decreasing the necessary cell mass and allowing long-term storage of competent cells. Finally, several red fluorescent protein reporters were assessed for their use in *R. ruber* C208. Together, these advances enable the development of more sophisticated synthetic biology tools for use in *R. ruber* C208 and other Actinobacteria.

2. Methods, Assumptions, and Procedures

2.1 Bacterial Strains and Growth Conditions

Rhodococcus ruber C208 (hereafter *R. ruber*) and the pNC903-TU1:sfGFP-DO plasmid were gifts from Dr Jimmy Gollihar at US Army Combat Capabilities Development Command (DEVCOM) Army Research Laboratory (ARL) South, University of Texas at Austin. *R. ruber* was routinely cultured aerobically on tryptic soy broth (TSB) or tryptic soy agar (TSA) (BD Difco) at 28–30 °C. Colonies formed after 2–3 days and continued to develop morphologically over 2 weeks. Growth in TSB was generally observed after 24–36 h, with a substantial portion of the cellular mass collected in an easily dispersed flocculent. *Escherichia coli* NEB10β cells were routinely cultivated at 37°C on Luria Bertani (LB) medium and used as the host strain for plasmids. Media were supplemented with chloramphenicol at 30 μg mL^{-1} or kanamycin when appropriate at 50 μg mL^{-1} for *E. coli* and 30 μg mL^{-1} for *R. ruber*.

2.2 Genome Sequencing

The *R. ruber* C208 genome was sequenced using a combination of short-read (iSeq100; Illumina) and long-read (MinION; Oxford Nanopore Technologies) sequencing. Genomic DNA was isolated using the EZNA Bacterial DNA Kit (Omega Biotek). Library preparation was performed with the Ligation Sequencing Kit (SQK-LSK109; Oxford Nanopore Technologies) for long-read sequencing and with the Nextera DNA Flex Library Prep Kit (Illumina) for 2 × 151 base pair paired-end short-read sequencing. Short-read sequencing was trimmed with trimmomatic (v.0.39),[23] and long-read sequencing was trimmed with porechop (v.0.2.4).[24] Low-quality reads were filtered from the long-read sequencing with NanoFilt (v.2.5.0).[25] Both short-reads and long-reads were used in the final assembly, which was performed with unicycler (v.0.4.7).[26] The final consensus was generated with medaka (v.0.10.0),[27] and prokka (v.1.14.5)[28] was used for the annotation.

2.3 Growth-Curve Measurements

Growth and culture pH of *R. ruber* was assessed in liquid media for three biological replicates in a 1-mL volume in 48-well microbioreactor flower plates (MTP-48-BOH2; m2p-Labs) using a BioLector II (m2p-Labs) at 30 °C, shaking at 250 rpm, with humidity maintained at 85% throughout the experiment. For growth measurements, absorbance readings for three independent uninoculated controls were averaged and subtracted from each individual time point to correct for background. Growth experiments were completed for two rich media—LB and TSB—and three minimal media—artificial medium (AM),[29] M9 Minimal Salts (BD Difco), and Yeast Nitrogen Base—without amino acids (YNB; BD Difco). Minimal media were supplemented with trace mineral supplements and trace vitamin supplements, both sourced from ATCC, at the manufacturer-recommended concentration for experiments using glucose (0.5% w/v) as the added carbon source. Growth of *R. ruber* was also characterized on M9 medium supplemented with 0.5% w/v or v/v sodium adipate, sodium benzoate, sodium 4-hydroxybenzoate, sodium protocatechuate, sodium *p*-coumerate, butanol, isopropanol, and succinate, all sourced from Sigma-Aldrich. Where sodium compounds were indicated, sodium hydroxide was used to convert the powdered acidic compounds to sodium salts to increase their solubility.

2.4 Antibiotic-Resistance Screening

The Minimum Inhibitory Concentration (MIC) of a panel of antibiotics against *R. ruber* was determined using liquid- and plate-based assays. Liquid-based assays were performed on TSB using three biological replicates in a 96-deep well format with a range of concentrations of ampicillin (12.5–400 μg mL^{-1}), chloramphenicol (3.75–120 μg mL^{-1}), erythromycin (6.25–200 μg mL^{-1}), gentamicin (3.75–120 μg mL^{-1}), kanamycin (6.25–200 μg mL^{-1}), spectinomycin (12.5–400 μg mL^{-1}), and tetracycline (2.5–80 μg mL^{-1}), all compounds from Sigma Aldrich. Cultures were inoculated at a 1:100 dilution factor, sealed with a Breathe-Easy film (Sigma Aldrich), and incubated at 28 °C while shaking at 220 rpm for 20–24 h. End-point OD_{600} readings were collected using a Biotek Synergy Neo2 plate reader with 100 μL of each culture that was transferred to a flat-bottom 96 well plate. The MIC was determined as the lowest concentration of antibiotic that prevented increase in OD_{600} above background.[30] For plate-based assays, 150 μL of a single biological replicate of a *R. ruber* culture grown to an OD_{600} >1.0 at 28 °C was spread evenly on a TSA plate. MIC gradient strips (Liofilchem) for each of the aforementioned antibiotics were placed onto the inoculated surface. Plates were subsequently incubated at 30 °C overnight. The MIC was determined by the indicated concentration where the ellipse of growth intersected the test strip.

2.5 Transformation

Electrocompetent *R. ruber* were prepared as described previously,[17] with the following modifications. A single colony of *R. ruber* was used to inoculate a TSB starter culture. After 32 h of growth at 28 °C, the starter culture was used to inoculate TSB containing 200 mM of glycine and 44 mM of sucrose at a 1:200 ratio. Following overnight growth at 28 °C, *R. ruber* cells were pelleted and washed three times with one-quarter of the original volume of ice-cold 10% glycerol. Remaining cells were resuspended in one-hundredth of the original volume and distributed into 50-µL aliquots. Aliquots were either snap frozen and stored at –80 °C for the indicated amount of days or used immediately for electroporation. Individual aliquots were added to a prechilled 1-mm gap-width electroporation cuvette (Bio-Rad) with 200 ng of plasmid DNA. Reactions were incubated for 5 min on ice prior to electroporation at 1.25 kV, 25 µF, and 200 Ω with a Bio-Rad Gene Pulser Xcell Electroporation System. Cells were recovered for 3–4 h in TSB at 28 °C prior to plating on LB or TSA containing 30 µg mL^{-1} of kanamycin.

2.6 Golden Gate Assembly

Plasmids carrying the genes encoding the pConstitutive promoter (PSlivStr)[19] and E2-Crimson, mCherry, and mScarlet fluorescent proteins, each flanked by BsaI restriction sites and unique overhangs for Golden Gate cloning,[31] were gifts from Dr Gollihar. Purified plasmids containing these parts were assembled in Golden Gate cloning reactions using Eco3I (ThermoFisher) and T7 DNA ligase (New England Biolabs [NEB]). Thirty percent of each Golden Gate reaction was transformed into chemically competent NEB10β *E. coli*. Kanamycin-resistant colonies expressing the fluorescent protein of interest were isolated, and the sequence-verified plasmids obtained from these colonies were used to transform *R. ruber*. The list of plasmids used in this study can be found in Table 1.

Table 1 Plasmids used in this study

Plasmid	Description	Reference
pGG0-AmpR:PSlivStr	BsaI flanked pConstitutive promoter (PSlivStr) in pGG0-AmpR	19, this work
pGG0-AmpR:E2-Crimson	BsaI flanked E2-Crimson in pGG0-AmpR	This work
pGG0-AmpR:mCherry	BsaI flanked mCherry in pGG0-AmpR	This work
pGG0-AmpR:mScarlet	BsaI flanked mScarlet in pGG0-AmpR	This work
pNC903-TU1:sfGFP-DO	pNC903 *Rhodococcus* origin, ColE1 *E. coli* origin, Kan^R and pYTK047 GFP dropout.	31, 32, this work
pNC903-TU1:PSlivStr-E2-Crimson	E2-Crimson expressed from the pConstitutive promoter in pNC903-TU1	This work
pNC903-TU1:PSlivStr-mCherry	mCherry expressed from the pConstitutive promoter in pNC903-TU1	This work
pNC903-TU1:PSlivStr-mScarlet	mScarlet expressed from the pConstitutive promoter in pNC903-TU1	This work

2.7 Flow Cytometry

Flow cytometry was performed using a Sony Spectral Cell Analyzer SA 3800. The system was equilibrated into NERL Blood Bank Saline (ThermoFisher). *R. ruber* cultures were grown to mid-log phase and diluted to 3% of their original concentration in sterile phosphate-buffered saline (PBS) prior to analysis. A threshold of 2% was set for the forward scatter with a gain of 12%. The side-scatter voltage was set at 27%, and the fluorescence photomultiplier tube voltage was set at 52% for E2-Crimson and mScarlet and 58% for mCherry. Data was collected for 10,000 events for the gate appropriate for each fluorescent protein. Wild-type *R. ruber* was used as a negative control to normalize the fluorescence signal for each reporter. Flow-cytometry data was processed using FlowCal to calculate the geometric mean for each population.[33]

3. Results

3.1 Genome Sequence of *R. ruber* C208

We used a combined Illumina iSeq100 short-read and Oxford Nanopore Technologies MinION long-read sequencing approach to generate the *R. ruber* C208 genome sequence. The combined reads assembled into a 5,547,825 base-pair chromosome with 70.5% guanine–cytosine (GC) content and a 165,987 base-pair plasmid with 68% GC-content. Notably, only a single plasmid was assembled for *R. ruber* C208, in contrast to other sequenced strains of *R. ruber* where either zero or two plasmids were sequenced.[9,10] Based on size, the single assembled plasmid in *R. ruber* C208 appears to correspond to the pYYL1.1 in *R. ruber* YYL[34] and the

"unnamed1" plasmid in *R. ruber* R1.[9] A draft annotation of both the genome and plasmid were generated using prokka (v.1.14.5).[28]

3.2 BioLector Microbioreactor Enables *R. ruber* Growth Measurements

We grew *R. ruber* in two rich media (LB and TSB) and three minimal media (M9, YNB, and AM) that were supplemented with 0.5% w/v glucose as a carbon source to identify optimal experimental growth conditions, as depicted in Fig. 1. As *R. ruber* was observed to flocculate in liquid culture during initial experiments (data not shown), 48-well microbioreactor flower plates were used to generate growth curves in a BioLector II. The flower-plate design allowed for increased dispersion and more accurate readings compared with tubes or 96-well microplates, while providing the ability to also monitor culture pH. *R. ruber* grew to a significantly higher cell density in TSB than in LB (Fig. 1a), or any minimal medium tested (Fig. 1c). *R. ruber* grew better in M9 supplemented with glucose than in either of the other two minimal media tested, with YNB resulting in very low growth (Fig. 1c). The culture's pH increased over time in the two rich media tested compared with the uninoculated controls (Fig. 1b), while the culture pH decreased over the course of the experiment in each of the minimal media tested (Fig. 1d). The mean and standard error of the mean (SEM) of three independent biological replicates are depicted in Fig. 1. These results suggest that TSB and M9 media are suitable for cultivation of *R. ruber*.

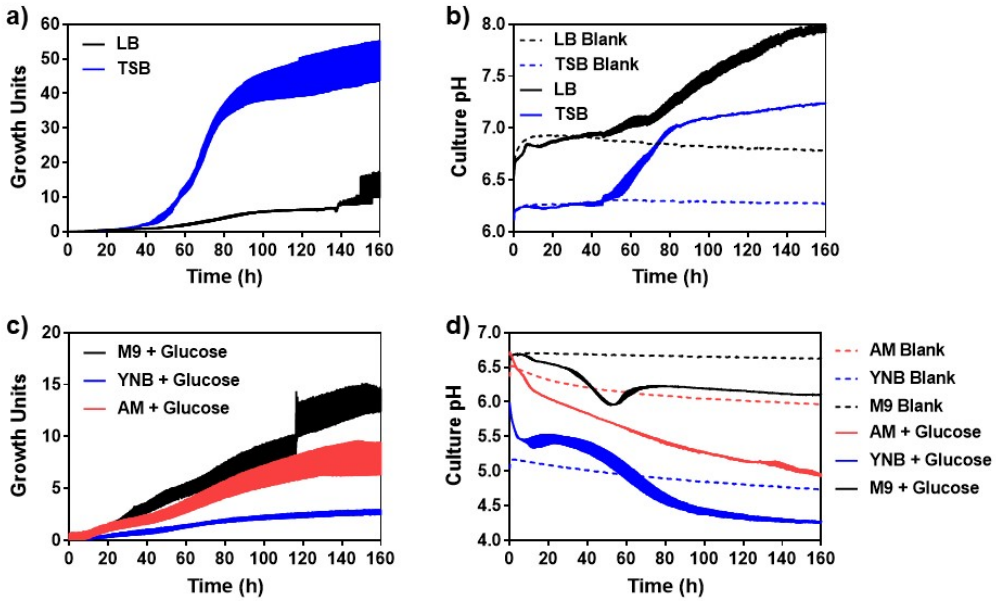

Fig. 1 ***R. ruber*** **growth and culture pH on the a) and b) LB and TSB rich media and the c) and d) M9, YNB, or AM media supplemented with 0.5% glucose**

3.3 *R. ruber* Grows on Diverse Carbon Sources

R. ruber was originally isolated due to its ability to survive on polyethylene as a sole carbon source,[5] and other *R. ruber* strains can grow on a variety of aromatic carbon sources.[9–12] Therefore, we examined the growth of *R. ruber* in M9 medium supplemented with alcohol (isopropanol and butanol), dicarboxylate (succinate and adipate), and aromatic (benzoate, 4-hydroxybenzoate, protocatechuate, and *p*-coumerate) carbon sources in our microbioreactor system, as depicted in Fig. 2. *R. ruber* grew in each of the carbon sources tested (Fig. 2). Growth proceeded most rapidly on succinate (Fig. 2a), butanol (Fig. 2b), and 4-hydroxybenzoate (Fig. 2c), while there was approximately a 120-h lag phase observed before growth proceeded on adipate (Fig. 2a). The mean and SEM of three independent biological replicates are depicted in Fig. 2. These results indicate that *R. ruber* is capable of degrading a variety of carbon sources.

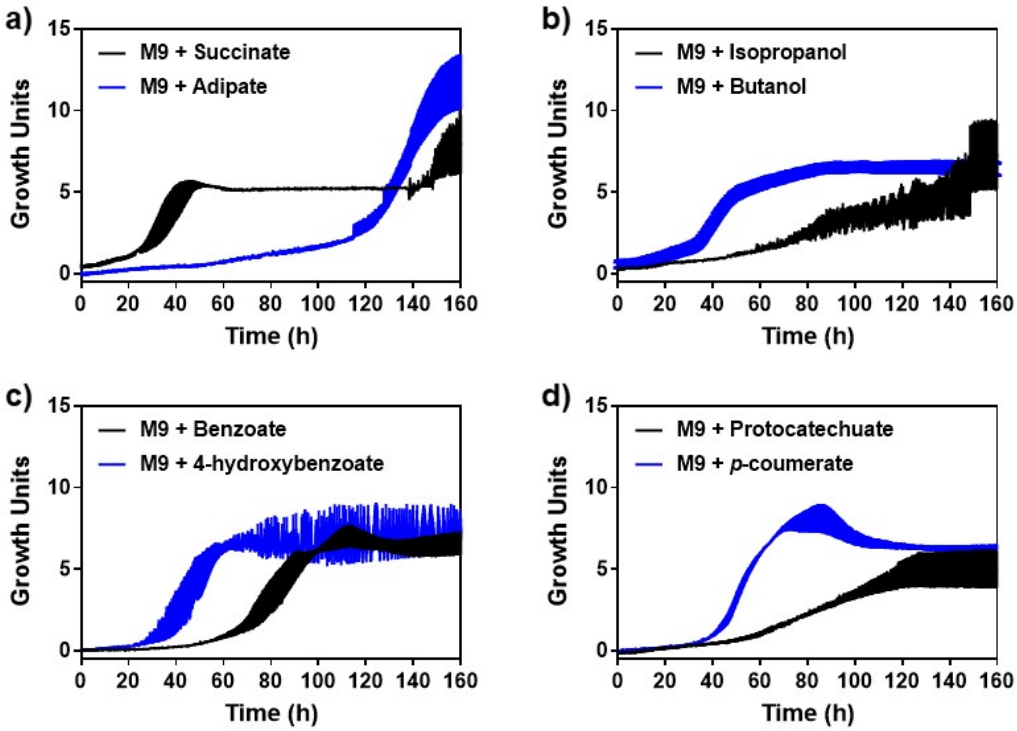

Fig. 2 ***R. ruber*** **growth on M9 minimal media containing 0.5% of a) succinate or adipate, b) isopropanol or butanol, c) benzoate or 4-hydroxybenzoate, and d) protocatechuate or *p*-coumerate**

3.4 *R. ruber* is Sensitive to Common Laboratory Antibiotics

Antibiotic-resistance genes are useful selectable markers for the introduction of foreign DNA to cells, but they require low background resistance to the targeted

antibiotic for proper selection. We sought to determine the antibiotic sensitivity of *R. ruber* growing on TSB to a panel of common laboratory antibiotics by determining the MIC of *R. ruber* growth in plate- and liquid-based assays. *R. ruber* was somewhat resistant to streptomycin, with a calculated MIC of 48 μg mL^{-1} (Table 2). *R. ruber* was sensitive (MIC ≤ 1.5 μg mL^{-1}) to all other antibiotics tested in the plate-based assay, with the greatest sensitivity to chloramphenicol and gentamicin, where no growth was observed in proximity to the test strip (Table 2). Similar results were observed in the liquid-based MIC assay, with growth above background only observed for spectinomycin up to 50 μg mL^{-1} and the two lowest concentrations of chloramphenicol (3.75 and 7.5 μg mL^{-1}), as shown in Table 2. Liquid MICs are listed in Table 2 as the lowest concentration where no growth was observed above the background and the next lowest concentration (or less than, if no lower concentration was tested). Liquid MICs were determined for three independent biological replicates and plate MICs were determined for a single biological replicate. These results indicate multiple commonly used antibiotic cassettes could be appropriate for use in *R. ruber*.

Table 2 MIC of common laboratory antibiotics for *R. ruber*

Antibiotic	Plate MIC	Liquid MIC
Ampicillin	0.25	<12.5
Chloramphenicol	S	7.5–15
Erythromycin	1	<12.5
Gentamicin	S	<3.75
Kanamycin	1.5	<6.25
Spectinomycin	48	50–100
Tetracycline	1	<6.25

3.5 Electroporation of *R. ruber*

The ability to easily introduce foreign DNA to cells via transformation is a requirement for development as a synthetic biology chassis.[35] General transformation protocols via electroporation exist for the introduction of DNA to *R. ruber*[36] and other *Rhodococcus* species,[17,37] but they require a significant amount of cell mass and exogenous DNA. We sought to simplify and increase the efficiency of these electroporation methods by using a small gap-width cuvette with decreased amounts of cells and DNA, while also determining the duration that cells could maintain competence and viability at –80 °C. A previously described protocol[17] was modified to use a 1-mm-gap-width cuvette, while maintaining the voltage to gap-width ratio and decreasing the concentration of plasmid to cell-volume ratio.

R. ruber was transformed with the pNC903-TU1:sfGFP-DO plasmid using the modified protocol either immediately after preparation or after the indicated

amount of time stored at –80 °C (Fig. 3). Transformations were spot-plated on kanamycin selective media at the indicated dilutions from 0.5 mL of recovery medium. The remaining cells were concentrated and spread on the other half of the plate, as depicted in Fig. 3. Kanamycin-resistant *R. ruber* colonies expressed superfolder green fluorescent protein (sfGFP) as visualized using an iBright CL 1500 Imaging system (Fig. 3), although the green coloration was not bright enough to be seen by eye or with the aid of an ultraviolet flashlight (data not shown). Electrocompetent cells exhibited approximately a twofold decrease in transformation efficiency after storage at –80 °C, but maintained that level of efficiency for at least four months (Fig. 3). These results indicate that efficient transformation of *R. ruber* with low amounts of DNA and cell mass is possible, without the need to freshly prepare competent cells.

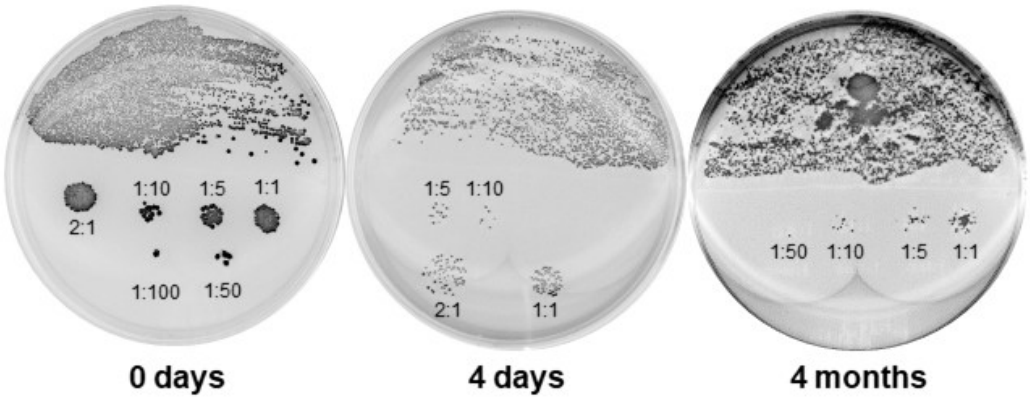

Fig. 3 ***R. ruber*** **electrocompetent cells maintain high efficiency**

3.6 *R. ruber* Highly Expresses Red Fluorescent Proteins

Rhodococcus colonies and cultures mature to a light-orange color due to the production of carotenoid compounds.[38] As the expression of sfGFP from the *E. coli glpT* promoter on the pNC903TU1:sfGFP-DO plasmid was poor in *R. ruber* (Fig. 3), we sought to assess the signal produced by red fluorescent proteins under the control of a strong constitutive *Rhodococcus* promoter.[19] The fluorescent proteins E2-Crimson, mCherry, and mScarlet were cloned into the pNC903TU1:sfGFP-DO plasmid by Golden Gate assembly[31] under the control of the pConstitutive promoter originally identified in *Streptomyces lividans* TK24.[19,39] Expression of all three fluorescent proteins produced a visible color change to *R. ruber* colonies when grown on solid media (Fig. 4a). Moreover, *R. ruber* expressing these fluorescent proteins could be detected via flow cytometry, with mCherry and mScarlet producing substantially stronger signals than E2-Crimson (Fig. 4b). Figure 4b depicts the geometric mean fluorescence intensity for each of

the three strains in Fig. 4a, as determined by flow cytometry in the measurement channel for each fluorescent protein. Figure 4b graphs the mean and standard deviation of the three independent biological replicates. These results suggest that red fluorescent proteins, particularly mCherry and mScarlet, are suitable reporter genes in *R. ruber* for the testing of promoter and ribosome-binding site strength, as well as other applications.

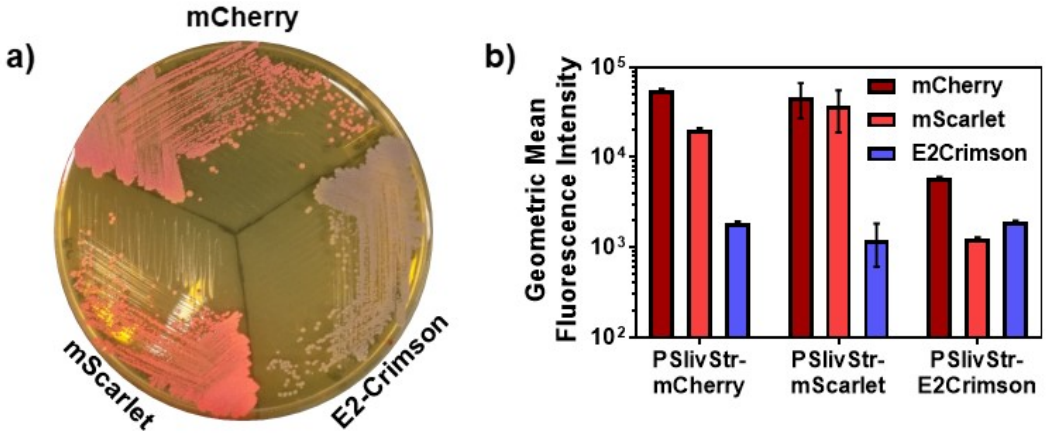

Fig. 4 Expression of the red-fluorescent proteins mCherry, E2-Crimson, and mScarlet by *R. ruber* a) on solid media and b) measured via flow cytometry

4. Discussion

4.1 *R. ruber* Annotated Genome

The *R. ruber* genome was sequenced with a combined short-read and long-read approach to enable assembly and draft annotation of its chromosome and a single plasmid. The size and GC-content of both the chromosome and plasmid were consistent with previously assembled *R. ruber* genome sequences.[9,10] Unlike previously sequenced *R. ruber* strains, our sequencing of *R. ruber* C208 revealed the presence of only a single plasmid. It is possible this is an artifact of our sequencing due to the loss of a second plasmid during propagation, insufficient isolation of plasmid DNA, or sequencing of an insufficient number of reads to assemble the plasmid. However, it is also possible that *R. ruber* only carries one plasmid, as *Rhodococcus spp.* harbor a variable number of circular and linear plasmids.[9,40–42]

Draft annotation of *R. ruber* genes enables the identification of potential genomic integration sites and gene functions that can be harnessed for synthetic biology. For example, overexpression of the native TatAC complex increased secretion of a laccase with a native TatAC-dependent secretion signal in *R. opacus* PD630,[43] and

integration of genes into the chromosome can remove the need for continuous antibiotic selection.[35] In an effort to replicate the overexpression of TatAC, the draft annotated *R. ruber* putative TatA and TatC protein sequences were compared to those of published *Rhodococcus* TatA and TatC sequences. This effort unveiled two errors with the draft annotation and assembly. First, the draft annotation incorrectly identified the start codon for TatA, predicting a putative protein 357 amino acids longer than an annotation that would be in agreement with other published genome sequences (data not shown). Second, an extra guanine was added to a poly-guanine sequence in the assembly, which would have indicated a frameshift in the TatC open reading frame. This guanine insertion was determined to be an assembly error by Sanger sequencing of the DNA in question (data not shown). Together, these examples indicate that the assembled and annotated *R. ruber* genome will require additional verification to use in the design of future experiments. An important step in that verification will be the examination of the *R. ruber* transcriptome via RNASeq and proteome via mass spectrometry in varied media conditions. These experiments will identify putative transcriptional start sites for individual genes and the proteins that are produced from them.

4.2 Hydrophobic Growth of *R. ruber*

When *R. ruber* was grown in polypropylene culture tubes, a substantial portion of the cell mass flocculated (data not shown). *R. ruber* flocculation did not interfere with the ability to grow bulk liquid cultures for use in the preparation of electrocompetent cells or MIC determination; however, flocculation also occurred in 96-well microplate cultures that were initially attempted to measure the growth of *R. ruber* in rich and minimal media (data not shown). Flocculation led to highly variable absorbance readings from our plate reader, causing the data to be very difficult to interpret. We overcame this issue through the use of flowered 48-well plates in a BioLector II microbioreactor (Figs. 2 and 3), which more readily dispersed the flocculants and gave more consistent growth measurements. Flocculation of *Rhodococcus* strains is thought to be caused by cell-wall mycolic acids and extracellular polysaccharides that lead to hydrophobic growth in liquid culture and a rough colony phenotype.[44,45] Moreover, flocculation is associated with degradation of alkanes in *R. erythropolis*.[46] As the flocculation phenotype could be significant to the potential uses of *R. ruber* for bioremediation, it is important to maintain it during laboratory experiments. Thus, there are limitations to the potential assays that can be accurately performed to screen additional phenotypes in *R. ruber*, and care should be taken to design future experiments where *R. ruber* flocculation does not interfere with measurements.

4.3 Degradative Potential of *R. ruber*

R. ruber was capable of utilizing all tested alcohols (isopropanol and butanol), aromatics (benzoate, 4-hydroxybenzoate, protocatechuate, and *p*-coumerate), and dicarboxylates (succinate and adipate) as sole carbon sources (Fig. 2). Other *Rhodococcus* strains have been described to harbor the β-ketoadipate pathway, which enables the breakdown of aromatic compounds that include benzoic acid, 4-hydroxybenzoic acid, protocatechuic acid, and *p*-coumeric acid.[47] Similarly, *Rhodococcus erythropolis* DCL14 was reported to degrade C5–C16 hydrocarbons and C1–C12 alcohols, including up to 2% butanol, as a sole carbon source.[48] Succinate is an intermediate in the tricarboxylic acid cycle, allowing it to function as a carbon source for many bacterial species. *Rhodococcus rhodochorus* breaks down adipate as an intermediate in ε-caprolactam degradation.[49,50] Adipate and ε-caprolactam are monomer precursors for the synthesis of nylon that are found in waste from these processes,[49] underscoring the bioremediation potential of *R. ruber* and related Actinobacteria species.

4.4 Red Fluorescent Proteins as Reporters in *R. ruber*

R. ruber naturally forms light-orange colonies due to the production of carotenoid compounds.[38] The red-fluorescent proteins E2-Crimson, mCherry, and mScarlet can be expressed to levels that visibly change the colony color (Fig. 4a). All three of these fluorescent proteins could also be detected by flow cytometry, but the fluorescence intensity of E2-Crimson was considerably lower in *R. ruber* than that of mCherry or mScarlet (Fig. 4b). Moreover, background levels of fluorescence by mCherry and mScarlet in the E2-Crimson channel were approximately equal to the fluorescence intensity of E2-Crimson, suggesting these fluorescent proteins would not be suitable for two-color flow cytometry. Similarly, mScarlet exhibited high background signal in the mCherry channel, which would prevent mCherry and mScarlet from being used together. Thus, testing of additional fluorescent proteins in the green–blue spectrum will be needed before two-color flow cytometry can be used successfully in *R. ruber*.

5. Conclusions

Here, we demonstrate an experimental synthetic biology pipeline to domesticate a bacterial chassis organism using the Actinobacteria species *R. ruber* C208 as an example. Determining the appropriate growth media, antibiotic-resistance cassettes, transformation protocol, and reporter proteins for use in a given chassis organism enables the design of more sophisticated synthetic biology experiments. The unique metabolic capabilities of *R. ruber* C208 and other Actinobacteria

species coupled with the domestication of these species as synthetic biology chassis organisms makes them promising candidates for bioremediation and/or biomanufacturing of Army-relevant materials.

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List of Symbols, Abbreviations, and Acronyms

AM	artificial minimal medium
ARL	Army Research Laboratory
DEVCOM	US Army Combat Capabilities Development Command
DNA	deoxyribonucleic acid
GC	guanine–cytosine
LB	Luria Bertani medium
LDPE	low-density polyethylene
MIC	minimum inhibitory concentration
MRE	meal, ready-to-eat
NEB	New England Biolabs
PBS	phosphate-buffered saline
R. ruber	*Rhodococcus ruber* C208
SEM	standard error of the mean
sfGFP	superfolder green fluorescent protein
spp.	species
TSA	tryptic soy agar
TSB	tryptic soy broth
w/v	weight per volume
v/v	volume per volume
YNB	yeast nitrogen base

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