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Protocol to develop a synthetic biology toolkit for the non-model bacterium *R. palustris*

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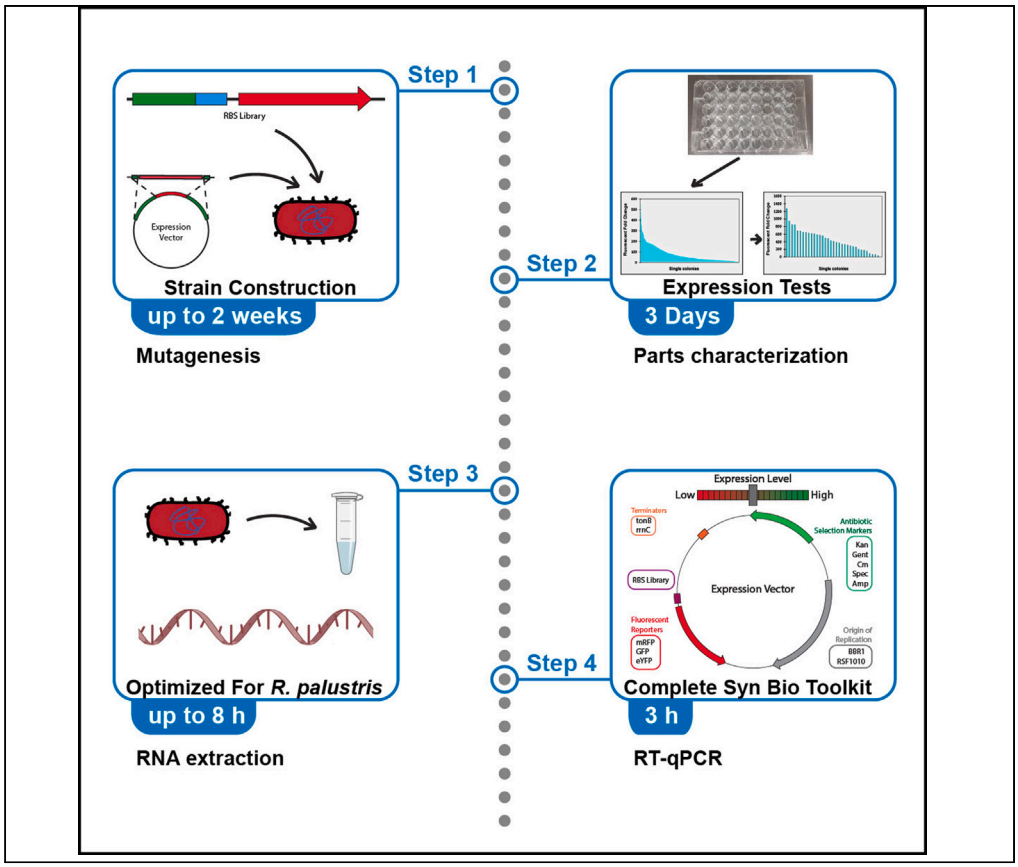

Numerous biology tools are developed to work for model organisms, which however do not work effectively in non-model organisms. Here, we present a protocol for developing a synthetic biology toolkit for *Rhodopseudomonas palustris* CGA009, a non-model bacterium with unique metabolic properties. We describe steps for introducing and characterizing biological devices in non-model bacteria, such as the utilization of fluorescence markers and RT-qPCR. This protocol may also be applicable for other non-model organisms.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol applicable for characterizing biological devices in non-model organisms

Optimized synthetic biology toolbox for heterologous gene expression in *R. palustris*

Steps for incorporating heterologous genes into *R. palustris*' endogenous plasmid

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Protocol to develop a synthetic biology toolkit for the non-model bacterium *R. palustris*

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SUMMARY

Numerous biology tools are developed to work for model organisms, which, however, do not work effectively in non-model organisms. Here, we present a protocol for developing a synthetic biology toolkit for *Rhodopseudomonas palustris* CGA009, a non-model bacterium with unique metabolic properties. We describe steps for introducing and characterizing biological devices in non-model bacteria, such as the utilization of fluorescence markers and RT-qPCR. This protocol may also be applicable for other non-model organisms.

For complete details on the use and execution of this protocol, please refer to Immethun et al..[1]

BEFORE YOU BEGIN

This protocol outlines a procedure by which expression systems can be optimized within *Rhodopseudomonas palustris* CGA009. This protocol contains a methodology which can be applied to other non-model organisms. The strengths of various regulatory parts, selection markers, and fluorescent reporters have been characterized using the methods in this protocol. An optimized procedure for extracting RNA from *R. palustris* is provided here, as well as procedures for measuring expression levels through both fluorescence intensity and RT-qPCR. A method of inserting expression cassettes into *R. palustris*' endogenous plasmid is also included in this protocol. Refer to the key resources table for a complete list of reagents and tools.

Preparation of photosynthetic media (PM), 112 Van Niel's media (VN), LB and solid media

Timing: 4 h

Recipes for the various media can be found in the materials and equipment section.

Note: Recipe for LB media can be found on LB Broth, Miller container.

1. Add reagents in the order shown to a 2 L bottle.
   a. For PM media, adjust the pH using a pH meter by addition of HCl or NaOH 1 h after addition of nitrilotriacetic acid.
   b. Allow each reagent to completely dissolve before adding the next.
2. After adding all reagents required for liquid media and before adding reagents required for solid media, adjust pH to 7.0.
   a. If making PM, adjust pH instead to 6.8.
3. Sterilize media using an autoclave.

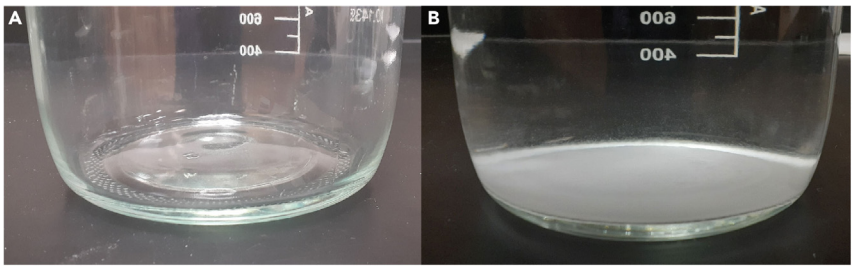

Figure 1. Photosynthetic media

(A) Photosynthetic media (PM) before being autoclaved.

(B) PM after being autoclaved. A white precipitate forms in the solution that must be resuspended before the addition of PM to any inoculation media.

a. After autoclaving PM media, an insoluble white precipitate forms as seen in Figure 1. When incubating *R. palustris* cultures, this precipitate must be resuspended by mixing before addition of PM.

⚠ CRITICAL: After sterilization of PM, as with handling LB or VN media, it is important to only open each bottle in sterile environments.

Preparation of Hunter's "Metals 44"

Refer to materials and equipment for Hunter's "Metals 44" recipe.

Preparation of *R. palustris* growth media

Timing: 1 h–2 h

This preparation describes how PM is used with other nutrients such as acetate (carbon source), sodium bicarbonate (redox balance), and ammonium sulfate (nitrogen source), to create a rigorously defined minimal media for the growth and incubation of *R. palustris*.

Note: It is recommended to make 500 mM solutions of sodium acetate, sodium bicarbonate, and ammonium sulfate in 500 mL glass bottles and sterilize using sterile disposable bottle top filters into a second, autoclaved 500 mL bottle. Perform all additions and inoculations required in this preparation in a sterile environment.

Note: The corresponding formula found below is an example solution that prepares enough inoculated *R. palustris* growth media to fill ten 250 mL Erlenmeyer flasks with 50 mL of solution, assuming additions are made from the 500 mM nutrient stocks. Adjustments can be made accordingly depending on the amount of inoculated growth media required.

4. Add sodium acetate, ammonium sulfate, and sodium bicarbonate in the order shown to a 1 L glass Fisher bottle.
   a. If the desired strains of *R. palustris* contain plasmids with antibiotic selection markers, add antibiotic to the appropriate concentration.
5. Mix PM bottle to resuspend the white precipitate and add PM before adding cells.

⚠ CRITICAL: The nutrients must be diluted by the PM media before the addition of cells. Addition of cells before dilution can cause *R. palustris* cultures to typically have a longer lag phase before reaching exponential growth.

6. Add Cells.

Note: "Seed cultures" are cultures that are grown from cells directly streaked from plates, it is advised that seed cultures of *R. palustris* are grown and reach the late exponential growth phase / stationary phase (~5 days) before dilution to OD_{660} = 0.2 and performing experiments with *R. palustris*.

a. For seed cultures, using a disposable inoculating loop, scrape cells from *R. palustris* plates and mix cells thoroughly into solution by pipetting up and down. *R. palustris* cells can remain aggregated if not mixed thoroughly.
b. For dilutions of seed cultures, measure OD_{660} of seed cultures using a UV-VIS Spectrophotometer and calculate the amount of seed culture required to dilute to OD_{660} = 0.2.

Note: The below formula assumes a seed culture OD_{660} = 0.8. Adjustments can be made as necessary using PM as balance.

c. Add 50 mL of inoculated growth media to 250 mL Erlenmeyer flasks.
d. Plug the top of each Erlenmeyer flask with autoclaved foam plugs to maintain a monoculture and aerobic conditions during incubation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Rhodopseudomonas palustris (Molisch) van Niel BAA-98™	ATCC	ATCCBAA-98
NEB® 10-beta competent *Escherichia coli* (E. coli DH10β)	New England Biolabs® Inc.	NEB#C3019I
Chemicals, peptides, and recombinant proteins		
LB Broth, Miller	Fisher Bioreagents	CAT#BP9723-2
Yeast extract	Fisher Bioreagents	CAT#BP9727-2
Nitrilotriacetic acid	Acros Organics	CAT#AC415742500
Magnesium sulfate (MgSO4)	Sigma-Aldrich	CAS#7487-88-9
Calcium chloride (CaCl2)	Sigma-Aldrich	CAS#10043-52-4
Ammonium molybdate tetrahydrate ((NH4)6Mo7O24 * 4H20)	Chem Implex International Incorporated	00021
Zinc sulfate heptahydrate (ZnSO4 * 7H2O)	Sigma-Aldrich	CAS#7446-20-0
Iron(II) sulfate heptahydrate (FeSO4 * 7H2O)	Acros Organics	CAT#AC423730050
Manganese sulfate monohydrate (MnSO4 *H2O)	Sigma-Aldrich	CAS#10034-96-5
Copper(II) sulfate pentahydrate (CuSO4 * 5H2O)	Sigma-Aldrich	CAS#7758-99-8
Cobalt(II) nitrate hexahydrate (Co(NO3)2 * 6H2O)	Sigma-Aldrich	CAS#10026-22-9
Sodium tetraborate decahydrate (Na2B4O7 * 10H2O)	Sigma-Aldrich	CAS#1303-96-4
Potassium phosphate monobasic anhydrous (KH2PO4)	Fisher Bioreagents	CAT#AC212590025
Sodium phosphate monobasic anhydrous (NaH2PO4)	Fisher Bioreagents	CAT#S397-500
Potassium phosphate dibasic anhydrous (K2HPO4)	Fisher Bioreagents	CAT#P290-500
Sodium phosphate dibasic anhydrous (Na2HPO4)	Fisher Bioreagents	CAT#S375-500
4-Aminobenzoic acid	Fisher Bioreagents	CAT#AAA1267330
Agar	Labscientific. inc	CAT#A466
Sodium succinate hexahydrate	Alfa Aesar	CAT#41983

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Trizol	Invitrogen™	CAT#15596018
RNAlater	Invitrogen™	CAT#AM7020
Chloroform	Alfa Aesar®	CAS#67-66-3
Methanol	Fisher Chemical™	A412-500
Agarose	Fisher Bioreagents	BP160-500
SYBR™ Safe DNA Gel Stain	Invitrogen™	S33102
Tris base	Chem Cruz	CAT#sc-3715B
Disodium EDTA salt dihydrate	Sigma-Aldrich	CAT#E5134
Glycerol	Research Products International	CAT#50488668
iso-Propyl alcohol	Supelco	CAT#PX1830-4
Ethanol 200 proof	Decon Labs, Inc.	CAT#2701
Glacial acetic acid	Fisher Chemical	CAT# A465-1
Critical commercial assays		
Monarch DNA Gel Extraction Kit	New England Biolabs® Inc.	CAT#T1020L
PureLink™ Quick Plasmid Miniprep Kit	Thermo Fisher Scientific	CAT#K210011
TURBO DNA-free™ Kit	Invitrogen™	CAT#AM1907
TaqMan® Gold RT-PCR Kit	Invitrogen™	CAT#4311235
Software and algorithms		
De Novo DNA RBS Calculator (Predict Translation Rates)	Reis et al.[2] Cetnar et al.[3] Borujeni et al.[4–6] Salis et al.[7]	https://salislab.net/software/predict_rbs_calculator
Other		
Nalgene™ Rapid-Flow™ Sterile Disposable Bottle Top Filters with PES Membrane, 150 mL, 0.2 µm pore, 45 mm neck	Thermo Scientific™	CAT# 595-4520
Falcon® 14 mL round bottom polystyrene test tube, with snap cap, sterile, individually wrapped, 500/case	Corning	CAT# 352001
1,000 µL, filtered, low retention, pre-sterile pipette tips	VWR North American	CAT#76322-154
200 µL, filtered, low retention, pre-sterile pipette tips	VWR North American	CAT#76322-516
10 µL, filtered, low retention, pre-sterile pipette tips	VWR North American	CAT#76322-512
Fisherbrand™ Petri Dish, Slippable Lid 100 mm × 15 mm sterile, polystyrene	Fisher Scientific	CAT#FB0875713
Fisherbrand™ reusable glass media bottles with cap, 2 L, 1 L, 500 mL, 250 mL, 100 mL	Fisher Scientific	CAT# FB8002000 CAT# FB8001000 CAT# FB800500 CAT# FB800250
Falcon® 15 mL high clarity PP centrifuge tube, conical bottom, with dome seal screw cap	Corning	CAT#352096
Falcon® 50 mL high clarity PP centrifuge tube, conical bottom	Corning	CAT#325070
Tuttnauer 2540E Autoclave	Tuttnauer	SKU MFI-TTN-Autoclave-2540E
Fisherbrand™ disposable inoculating loops and needles, flexible needle and loop	Fisher Scientific	CAT#22-363-595
Genesys 10S UV-VIS Spectrophotometer	Thermo Scientific	CAT#14-385-401
JAECE Identi-Plugs 27–34 mm, 35–45 mm	JAECE Industries	CAT#L800-C CAT#L800-D
New Brunswick Innova® 42R	Eppendorf	CAT#M1335-0004
Electroporation cuvettes	Fisherbrand™	CAT#FB102
Micropulser™	Bio-Rad	CAT#1652100
CryoTube™ Vials	Thermo Scientific	CAT#377224
PCR tubes strips with attached caps	Bio-Rad	CAT#TBC0802
48 well suspension culture plate	Greiner Bio-One	CAT#677102
Breathe-Easy® gas permeable sealing membrane for microtiter plates	Diversified Biotech	CAT#BEM-1
Microplate, 96 Well, PS, F-Bottom (Chimney Well) µClear®, Black Med. Binding	Greiner Bio-One	CAT#655096

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Allegra X-30R Centrifuge	Beckman Coulter	https://www.beckman.com/landing/ppc/cent/benchtop/allegra?msclkid=6ba7b71a567d111fceb4d42f43839cc9&utm_source=bing&utm_medium=cpc&utm_campaign=Centrifugation%20NAO%20-%20Brand&utm_term=allegra%206r&utm_content=Centrifugation%20NAO%20-%20Brand%20Allegra
Microfuge® 20R Centrifuge	Beckman Coulter	CAT#B30140
Costar® 1.7 mL Low Binding Snap Cap Microcentrifuge Tube	Corning®	CAT#3213
NanoDrop One	Thermo Scientific™	CAT#ND-ONE-W
SYBR™ Safe DNA Gel Stain	Invitrogen™	CAT#S33102
S1000™ Thermal Cycler	Bio-Rad	https://www.bio-rad.com/en-us/product/s1000-thermal-cycler?ID=ab2cabb3-9299-489f-93fe-754e14301ac6
PowerUp™ SYBR™ Green Master Mix	Applied Biosystems™	CAT#:A25742
SpectraMax® i3x	Molecular Devices	https://www.moleculardevices.com/en/assets/promotion/br/real-discovery-starts-here?cmp=7014u000001RlxCAAW&utm_source=bing&utm_medium=cpc&utm_campaign=MPR-Brand&utm_adgroup=MPR-Brand-Spectramax-i%20Series&utm_location=97570&utm_keyword=spectramax%20i3x&utm_device=c&utm_devicemodel=&utm_placement=&utm_adpostion=&utm_target=&utm_network=o&utm_creative=&msclkid=fc4bf27acc631b006aa5914f7242a8ab
Fisher Accumet AB150 pH Benchtop Meter Kit	Fisher Scientific	CAT#13-636-AB150
Eppendorf Mastercycler Realplex	Eppendorf	SKUEppRP2
CryoTube™ Vials	Thermo Scientific	CAT#343958

MATERIALS AND EQUIPMENT

Hunter's "Metals 44"

Reagent	Final concentration	Amount
dH20	N/A	50 mL
$ZnSO_4 * 7H_2O$	1.15 mM	0.44 g
$FeSO_4 * 7H_2O$	4.81 mM	1.16 g
$MnSO_4 * H_2O$	0.6 mM	0.134 g
$CuSO_4 * 5H_2O$	161 nM	0.4 mg
$Co(NO_3)_2 * 6H_2O$	13.2 μM	0.004 g
$Na_2B_4O_7 * 10H_2O$	N/A	2 mL
Total	**N/A**	**100 mL**

Store at 4°C for up to one year.

Note: Add a few drops of concentrated H_2SO_4 to prevent the slow precipitation of heavy metal ions. The solution should turn lime green when finished.

Photosynthetic media (PM)

Reagent	Final concentration	Amount
dH20	N/A	1,400 mL
Nitrilotriacetic Acid	1.15 mM	0.44 g
$MgSO_4$	4.81 mM	1.16 g
$CaCl_2$	0.6 mM	0.134 g
$(NH_4)_6Mo_7O_{24} * 4H_2O$	161 nM	0.4 mg

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Continued		
Reagent	Final concentration	Amount
$FeSO_4$ * $7H_2O$	13.2 μM	0.004 g
Hunter's "Metals 44"	N/A	2 mL
KH_2PO_4	50 mM	13.6 g
Na_2HPO_4	50 mM	14.2 g
$Na_2S_2O_3$ * $5H_2O$	157 μM	0.078 g
4-aminobenzoic acid	150 μM	0.0412 g
Succinate (solid media)	22.8 mM	5.4 g
Agar (solid media)	15 g/L	30 g
Total	**N/A**	**2 L**
Store at 20°C–25°C for up to one year.		

112 Van Niel's media (VN)		
Reagent	Final concentration	Amount
dH20	N/A	1,000 mL
K_2HPO_4	1 g/L	2 g
$MgSO_4$	0.5 g/L	1 g
Yeast Extract	10 g/L	20 g
Total	**N/A**	**2 L**
Store at 20°C–25°C for up to one year.		

⚠ CRITICAL: As HCl and KOH are used in balancing pH in PM and VN media, a lab coat, gloves, and safety glasses should be worn at all times while handling these chemicals.

Inoculated *R. palustris* PM growth media		
Reagent	Final concentration	Amount
$Na_2C_2H_3O_2$	20 mM	20.8 mL
$(NH_4)_2SO_4$	15.2 mM	15.8 mL
$NaHCO_3$	10 mM	10.4 mL
Antibiotic (optional)	N/A	N/A
Cells	OD_{660} = 0.2	130 mL
PM	N/A	343 mL
Total	**N/A**	**520 mL**
To be used immediately after making at 30°C.		

Alternatives: Equipment and reagent alternatives.

- All measuring equipment such as the Genesys 10S UV-VIS Spectrophotometer, SpectraMax® i3x plate reader, and Eppendorf Mastercycler Realplex, can be replaced by equipment with similar performance characteristics.
- All chemicals and reagents can be replaced by corresponding chemicals from other suppliers with similar grade and purity.
- Other lab equipment such as New Brunswick Innova® 42R incubator, Eppendorf or Falcon tubes, inoculating loops, etc., can be replaced by other corresponding lab equipment with similar performance characteristics.

STEP-BY-STEP METHOD DETAILS

Strain handling and construction

Timing: 1–2 weeks

This step outlines incubation and growth conditions of both *R. palustris* and *E. coli* cultures. Conditions for long-term storage of strains are detailed here as well. Oligonucleotides used for constructing strains may be purchased from Eurofins Genomics or a similar supplier. All plasmids were initially constructed in 10-beta competent *E. coli*, and plasmid construction was carried out through either hot fusion or blunt end ligation methods.[8] This section of the protocol also details a method of constructing a Ribosome binding site (RBS) library, which is a collection of different RBS sequences which have different effective translation initiation rates, as well as a method of comparing predicted translation initiation rates with experimentally derived expression levels.

1. Strain Storage and Construction.
   a. *Rhodopseudomonas palustris* (Molisch) van Niel BAA-98, strain designation CGA009 (*R. palustris*), may be obtained from American Type Culture Collection.
      i. *R. palustris* cultures are stored long-term at a final concentration of 20% glycerol.
      ii. *R. palustris* cultures are grown in 112 Van Niel's media (VN) or photosynthetic media supplemented with appropriate antibiotics depending on selection markers.

   Note: It is advisable to determine which antibiotics are compatible with the non-model organism of interest by performing growth tests with varying concentrations of several antibiotics individually.

   b. NEB® 10-beta competent Escherichia coli (*E. coli* DH10β) was used for plasmid construction.
      i. *E. coli* cultures are stored long-term at a final concentration of 15% glycerol.
      ii. *E. coli* cultures are grown in LB media and supplemented with the appropriate antibiotic.

2. Evaluation of PCR Primers.

   Note: Before PCR is performed on DNA, or RT-qPCR is performed on RNA samples, it is advisable to first evaluate the primers that will be used for PCR with a Taq master mix with 100ng of DNA. Primer concentrations will need to be first determined through an initial evaluation to avoid fluorescence from non-specific binding of the polymerase (such as from primer dimers).

   a. Make two sets of GoTaq® PCR Master Mix reactions, one containing DNA template, one without, with differing concentrations of primers as described below.
   b. Using a thermal cycler, perform a GoTaq® PCR reaction using the settings below.
   c. On a 1× TAE 2% agarose gel, perform electrophoresis on PCR products.
   d. Reactions with primer concentrations that produce a band in reactions containing DNA but fail to produce a band in reactions without DNA may be considered for further RT-qPCR reactions (troubleshooting 1).

3. *E. coli* Transformation by Electroporation.
   a. Streak wild-type *E. coli* from frozen stock onto a solid LB media plate.
   b. Incubate at 37°C for 12–24 h.
   c. Add 4 mL of LB media to a 14 mL polystyrene round-bottom tube.
   d. Inoculate with DH10β *E. coli* from frozen glycerol stock.
   e. Incubate at 37°C and 275 rpm for 8–24 h.
   f. Dilute strains to 1/40th in a separate 14 mL polystyrene round-bottom tube and incubate for 1 h at 30°C and 275 rpm.

   Note: Each tube of diluted *E. coli* will support 2 total transformations.

   g. Wash the new culture by centrifuging at 3,000 × *g* for 15 min and resuspend in 4 mL of sterile water.
   h. Centrifuge again at 3,000 × *g* for 20 min and resuspend in 4 mL of sterile water.

i. Centrifuge at 3,000 × *g* for 25 min.
j. Resuspend *E. coli* pellet in 100 μL of sterile water.
k. Add 50 μL of *E. coli* suspension to a 0.2 cm electroporation cuvette and 2 μL of hot fusion[8] or blunt end ligation product.
l. Insert the electroporation cuvette into an electroporator and pulse the electroporation cuvette at 2.5 kV.
m. Immediately add 450 μL of LB solution to the cuvette.
n. Immediately decant cuvette into a sterile 14 mL polystyrene round-bottom tube.
o. Incubate for 1 h at 37°C and 275 rpm.

Note: It is advisable to record the time elapsed for each electroporation. Successful electroporations with ligation mixtures usually have ~5 ms pulse times. Lower pulse times typically result in reduced transformation efficiency or cause the electroporator to arc, killing cell cultures.

p. Streak the electroporated cells onto solid LB media plates supplemented with the appropriate antibiotic.
q. Incubate at 37°C for up to 18 h.
r. Pick single colonies from LB plates containing the transformed *E. coli* cells by suspension in 4 mL of liquid LB media supplemented with the appropriate antibiotic.
s. Incubate at 37°C at 275 rpm for 8–18 h.
t. Harvest 0.5 mL (or an appropriate amount depending on the cryogenic vial used) of each transformed *E. coli* culture and add to a cryogenic vial.

Note: Take care to leave room for expansion while freezing.

u. Add 0.5 mL of sterile 30% glycerol solution for a final concentration of 15% glycerol.
    i. Mix by inverting tubes several or pipetting up and down.
    ii. Store at −80°C for long-term storage.

Pause point: New *E. coli* strains can be stored at −80°C for several years, cultures may be restarted from frozen stock to harvest subsequent plasmids.

v. Using a Plasmid Miniprep Kit, harvest plasmids from the remaining culture.

Pause point: Eluted plasmids are typically stable at −20°C for several years.

w. Submit samples for sequencing to ensure that no mutations have occurred during the transformation process and that the plasmid assembled correctly.

4. *R. palustris* Transformation by Electroporation.
    a. Streak frozen wild-type *R. palustris* stock onto a solid PM plate and incubate at 30°C for 5 days or until a sufficient amount of culture appears on the plate.
    b. Add 4 mL of sterile VN to a 14 mL polystyrene round-bottom tube.
    c. Inoculate with streaked *R. palustris* culture plates.
    d. Incubate at 30°C and 275 rpm for 48 h.
    e. Dilute to OD_{660} = 0.2 into another tube of 4 mL sterile VN.
    f. Incubate for 18 h at 30°C and 275 rpm, or until OD_{660} = 0.6.

Note: Likewise with *E. coli* each tube will support 2 total transformations.

g. Perform the same washing steps (g – j) as from *E. coli* Transformation by Electroporation.

Note: *R. palustris* cell pellets are not as easily disturbed as *E. coli* cell pellets, though care should still be taken not to resuspend and remove cells.

h. Add 100ng of the previously miniprepped plasmid of interest assembled in *E. coli* to the 0.2 cm electroporation cuvette.

Note: The initial plasmid assembly in *E. coli* is necessary to construct the plasmid correctly and without mutations.

i. Electroporate the cuvette using the same settings as with *E. coli* electroporation (k).
j. Pulse the electroporation cuvette, and immediately add 450 μL of VN solution to the cuvette.
k. Immediately decant cuvette into a sterile 14 mL polystyrene round-bottom tube and incubate up to 24 h at 30°C and 275 rpm.
l. Streak transformed cultures onto solid VN media supplemented with the appropriate antibiotic.
m. Incubate plates at 30°C for up to one week after electroporation.

Note: Colonies may not begin to appear until typically 5 days after electroporation.

n. Pick single colonies onto a second VN plate supplemented with the appropriate antibiotic.
o. Incubate at 30°C for 5 days or until a sufficient amount of culture appears on the plate.
p. Inoculate from single-colony plates and grow in 4 mL of VN media for 48 h.
q. Make frozen stocks of *R. palustris* by adding 0.5 mL of transformed *R. palustris* cultures to cryogenic vials (or an appropriate amount depending the cryogenic vial used) and add 0.5 mL of sterile 40% glycerol for a final concentration of 20% glycerol.
r. Store at −80°C.

Pause point: New *R. palustris* strains can be stored at −80°C for several years, cultures may be restarted from frozen stock for later handling.

s. Harvest 50 μL of culture into PCR tubes and lyse at 100°C for 30 min.

Note: Plasmids from *R. palustris* cultures do not need to be extracted, cell lysate can be used in PCR sequencing reactions.

t. Perform PCR with *R. palustris* cell lysate using sequencing primers.
u. Submit samples for sequencing to ensure a lack of mutation and correct assembly.

5. RBS Library Construction.

Note: An RBS library can be constructed by designing a degenerate oligonucleotide such as the one depicted in Figure 2. A primer is first designed matching the sequence immediately before the RBS site. A poly "N" tag is then added onto the end where the RBS sequence is located. The poly "N" tag will allow the RBS to be replaced by all possible RBS sequences. A second primer should be designed matching the reverse complement of the sequence immediately after the RBS to allow for a blunt end ligation assembly.

a. Perform PCR of the plasmid with the degenerate oligonucleotide and its pair.
b. Treat PCR product with DpnI enzyme to digest any plasmid that did not undergo PCR.
c. Purify PCR product using a PCR purification kit.
d. Perform a Blunt-End Ligation of the purified PCR product.
e. Transform *E. coli* with the blunt end ligation product for initial assembly, miniprep, and transform *R. palustris* using the above protocol.

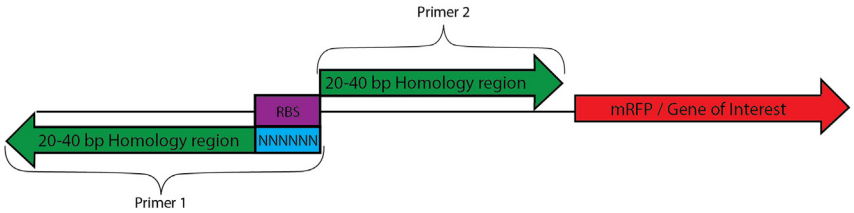

Figure 2. Degenerate oligonucleotide construction for saturation mutagenesis

Example of a degenerate oligonucleotide used to replace an RBS. The "N"s in each position for the RBS sequence allow for every possible combination of base sequences to replace the RBS. The poly "N" tag may be attached to either primer, but must be concatenated to them so as to replace the RBS sequence. Individual sequences can be obtained by performing blunt end ligation, transformation in *E. coli*, miniprep, and transformation in *R. palustris*. Picking single colonies will ensure that only individual RBS replacement sequences are present in any culture.

f. Pick a number of colonies proportional to the desired size of the RBS library onto separate solid VN media plates supplemented with the appropriate antibiotic.

Note: Multiple colonies can be picked onto a single plate as long as they are separated from each other.

Note: Depending on the number of base pairs of the RBS that are being replaced, a number of colonies should be picked. E.g., an RBS containing 6 base pairs that can be changed will result in 4096 unique possibilities. Only a subset should be picked as obtaining all possible sequences can be very difficult.

6. RBS Library Screening.
   a. Pick cells from each culture into separate wells of a 48 deep well plate.

Note: Each well should contain 500 μL of PM supplemented with sodium acetate, sodium bicarbonate, and ammonium sulfate nutrients. Incubate for 5 days at 275 rpm and 30°C.

   b. Transfer 200 μL of each culture to individual wells of a black 96 well plate.
   c. Measure fluorescence and absorbance of each culture and calculate expression fold change using the formula found in the fluorescent marker expression test section below.

Note: The RBS library at this point can be trimmed by excluding RBS sequences that have similar expression levels. One method of performing this is selecting sequences from discrete ranges of increased expression (e.g., out of 5 possible sequences that provide a fold change increase of 130–160, only 1 needs to be selected).

   d. Make frozen stocks of selected cultures by adding an equal volume of glycerol directly to each well of the 48 well plate.
   e. After adding glycerol to each well, seal the top of the plate with aluminum foil tops.
   f. Store at −80°C.

Pause point: RBS library cultures can be stored for several years at −80°C.

   g. Lyse 50 μL of each sample and use lysate for subsequent PCR for determining RBS sequences.
   h. Submit samples for sequencing to determine sequences for selected cultures.
   i. Obtain predicted translation rates for each RBS sequence using the Salis RBS Calculator found at https://salislab.net/software/predict_rbs_calculator.[2–7]
   j. Plot experimentally obtained expression rates vs predicted translation initiation rates for each sequence.

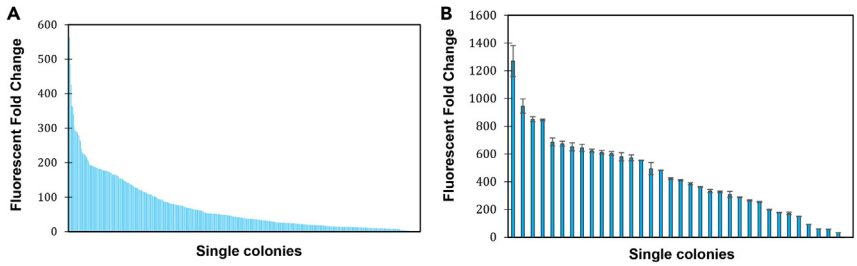

Figure 3. RBS library fluorescence scans

(A) Total single colonies picked from transformed *R. palustris* cultures with replaced RBS from Immethun and Kathol et al.[1] Fluorescence of each culture was determined by performing a fluorescence marker expression test and measured using a plate reader.

(B) Example of a trimmed RBS library. Trimming was performed by selecting several RBS's which provided the highest fluorescent intensities, then by selecting one RBS for each expression fold change threshold (~30 fold change).

Note: This plot will determine the efficacy of the calculator in predicting sequences with high translation initiation rates. Statistics such as R^2 or the Pearson coefficient can be used to determine the correlation between predicted and experimental values for different RBS sequences.

Note: Figure 3 from Immethun & Kathol et al.[1] depicts an example of a trimmed RBS library, where a linear gradient of expression level is made by selecting various RBS sequences with differing expression levels from an initial scan. Figure 3A shows the initial collection of colonies that were picked from plates, while Figure 3B depicts the trimmed RBS library.[1]

7. Genome Engineering by Double Homologous Recombination.
   a. A suicide plasmid is constructed by initially determining a plasmid origin of replication that is unable to replicate within *R. palustris*.

⚠ CRITICAL: It is important to choose a plasmid origin of replication that is compatible with *E. coli* DH10β, but not with *R. palustris* CGA009. Colony PCR using *R. palustris* lysate is used to determine which plasmid origins replicate in *R. palustris*. The p15A origin was selected for the study in Immethun & Kathol et al.[1] as it does not replicate in *R. palustris* but does within *E. coli* DH10β.

   b. Determine section of *R. palustris* genome for integration of the expression cassette.

Note: To ensure that the expression cassette replaces this specific site, the expression cassette is flanked by two homology arms (800–1,000 bp) which consist of the upstream and downstream regions of the open reading frame. These homology arms are designed to consist strictly of the DNA sequence of these regions as depicted in Figure 4.

   c. An expression cassette which includes a selection marker as well as a reporter gene, is added to the suicide plasmid via hot fusion method.[8]
   d. Transform the suicide plasmid into *E. coli* using the above protocol.
   e. Transform *R. palustris* using the sequence-verified plasmid after construction using *E. coli*.
   f. Verify integration into the genome via PCR.

Note: Two sets of primers are designed for this purpose, the first set should be designed to include a roughly 200 bp region before the 5′ homology arm, and the 200 bp region after the 3′ homology arm. The second set should include only the section of the genome that is being replaced. The first PCR reaction should produce a band which should be the added size of the expression cassette, the homology arms, and the ~200 bp sections before and after the 5′ and 3′ homology arms respectively. The second set of primers should not produce a band.

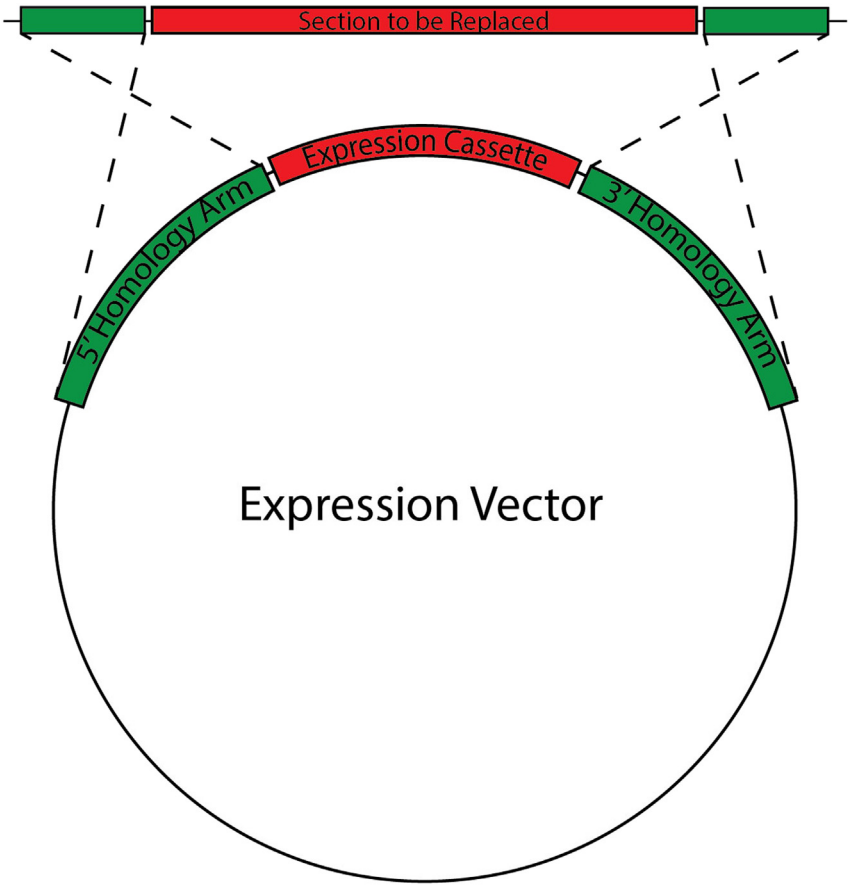

Figure 4. *R. palustris* double homologous recombination

An expression cassette consisting of a fluorescent reporter with its associated regulatory parts and an antibiotic resistance gene is flanked by two homology "arms" which comprise 800–1,000 bp of DNA sequence before and after the specified insertion point. The vector containing the expression cassette and homology arms contains the p15A origin, which has been verified to replicate in *E. coli* DH10β, but not in *R. palustris*. This ensures that transformed *R. palustris* cultures consist of strains that contain the expression cassette at the target site.

i. After verification of integration into the genome, perform a Fluorescent marker expression test to determine the level of expression from the integrated cassette.
ii. Flow cytometry may be subsequently performed to ensure homogeneity of the transformed culture of *R. palustris*.

8. Observing Plasmid Stability via Fluorescent Markers and Flow Cytometry.

Note: When transformation is performed on certain bacteria, whether they are model or non-model bacteria, it is possible that cells from picked colonies may not contain plasmids. Plasmids may also be lost over generations in bacterial cultures. Flow cytometry can be used in conjunction with fluorescent markers incorporated in these plasmids to observe their maintenance in bacteria. This protocol describes a method by which the proportion of cell cultures that have not retained the plasmid across generations can be determined.

a. Streak *R. palustris* cultures transformed with plasmids containing a fluorescent marker from frozen stocks onto solid VN plates supplemented with the appropriate antibiotic.

Note: For each flow cytometry experiment, wild-type *R. palustris* will be used as a negative control. Also streak an *R. palustris* culture transformed with a plasmid that is known to be maintained across several generations that also contains a fluorescent marker, this strain will act as a positive control.

b. Incubate *R. palustris* cultures at 30°C and 275 rpm for the desired amount of time.

Note: The desired amount of time is the length of time for which plasmid loss is expected to occur. For *R. palustris*, plasmid loss can occur over the course of a couple weeks. This can change depending on the strain of bacteria being handled. Several dilutions of the same culture may need to be performed as cultures can reach stationary phase multiple times before plasmid loss can occur.

c. Prepare samples for submission to a local flow cytometry core or facility.

⚠ CRITICAL: Bovine Serum Albumin (BSA) is sometimes added to samples of bacteria in order to reduce rejection rate in Flow Cytometry experiments. While it has not been observed for other possible fluorescent markers or other bacteria, the addition of BSA severely reduced the fluorescence of cells in *R. palustris* cultures with mRFP. As a result, it may be advisable to prepare two sample sets, one containing BSA and one without, to determine if BSA produces a "masking" effect on the fluorescence of various bacteria.

d. Using both the negative and positive controls, determine a threshold fluorescence intensity.

Note: Cells with fluorescence intensity higher than the threshold can be considered to contain the plasmid, while cells that have intensities lower than the threshold are considered not to contain the plasmid, such as in Figure 5.

e. Obtain proportions of cultures that have retained the plasmid.

Fluorescent marker expression test

Timing: 5 days

This procedure describes how the expression level of genes of interest in newly constructed strains of non-model bacteria can be measured. One of the most common methods of doing this is by inserting a fluorescent marker, such as monomeric red fluorescent protein or green fluorescent protein into a plasmid, which is then in turn inserted into bacterial cells. The use of fluorescent proteins is an alternative method to qPCR of determining expression levels, maintenance of plasmids, and strengths of regulatory parts such as promoters and RBS sequences. It is easier as it does not involve the extraction of RNA and subsequent RT-qPCR, which can be time-consuming and more expensive in terms of material costs. This procedure describes how the expression level of various fluorescent proteins is determined in *R. palustris.*

9. Fluorescence Expression Test.
   a. Streak *R. palustris* cultures transformed with plasmids of interest from frozen stocks onto solid VN plates supplemented with the appropriate antibiotic.

   Note: For each expression test, wild-type *R. palustris* will be used as a negative control.

   b. Incubate plates at 30°C and 275 rpm for 5–7 days.

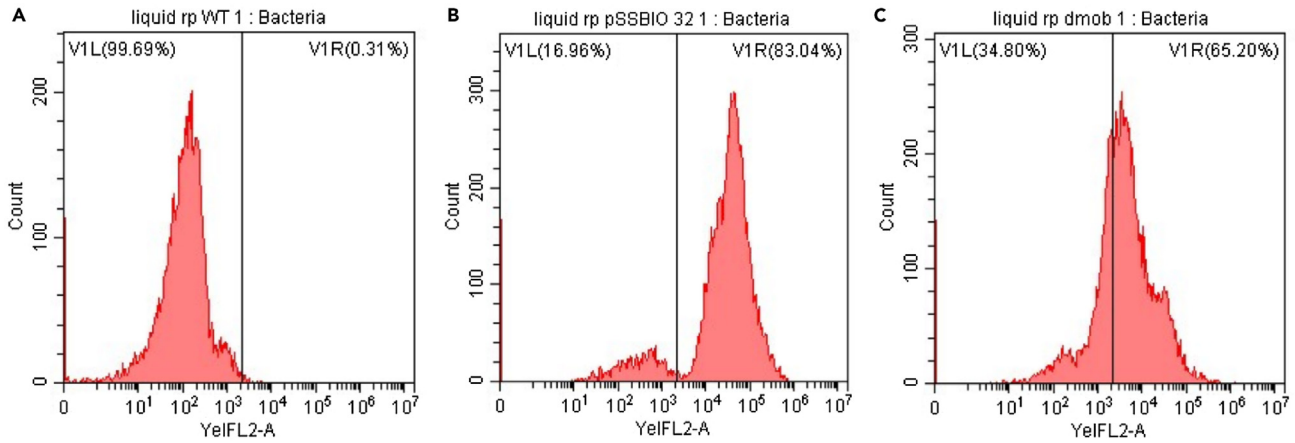

Figure 5. Flow Cytometry results of *R. palustris* strains

(A) An example of a negative control result of wild type *R. palustris*. This culture contains exclusively untransformed cells.

(B) An example of a result from a positive control strain of *R. palustris*. This strain is known to have a high plasmid maintenance and has a distinct fluorescence peak which can be distinguished from untransformed cells. The combination of a negative and positive control allows a fluorescence threshold to be determined to differentiate between transformed and untransformed cells.

(C) An example of an *R. palustris* culture with reduced plasmid maintenance. A smaller percentage of the culture has retained the plasmid than that of the positive control.

c. Prepare *R. palustris* "seed" culture in PM media for each strain of interest containing fluorescent proteins in 250 mL Erlenmeyer flasks.

Note: 1.5 mL of wild-type *R. palustris* culture will be needed for triplicate wells of negative controls in each plate.

d. Incubate *R. palustris* seed culture at 30°C and 275 rpm for 5 days.
e. Prepare dilutions of desired strains of *R. palustris* by preparing inoculated *R. palustris* growth media as described above (see materials and equipment) for 2 mL.

Note: Final concentrations of nutrients should remain the same and OD_{660} = 0.2, with PM used as balance.

f. Add 500 μL of inoculated growth media to 3 wells of a sterile 48 well suspension culture plate.
g. Cover the plate with a Breathe-Easy® Permeable Membrane to maintain both monoculture and aerobic conditions during incubation and incubate at 30°C and 275 rpm for 72 h. Figure 6 depicts an example of a prepared plate.

Note: Some incubator conditions can lead to evaporation of growth media during incubation, even through the permeable membrane. A beaker of sterile water may be placed in a safe position in the incubator to provide humidity and lessen evaporation effects.

h. Remove plate from incubator and add 200 μL of each culture to a black 96-well clear flat bottom plate (troubleshooting 2).
i. Add 200 μL of PM media in triplicate to act as a blank.
j. Measure fluorescence and absorbance of each culture using a plate reader (troubleshooting 3).

Note: Expression tests measuring fluorescence intensity of cultures using a plate reader can be performed by specifying the type of plate used in the measurement, the excitation and emission wavelengths of the fluorescent protein of interest, read area on the plate, as well

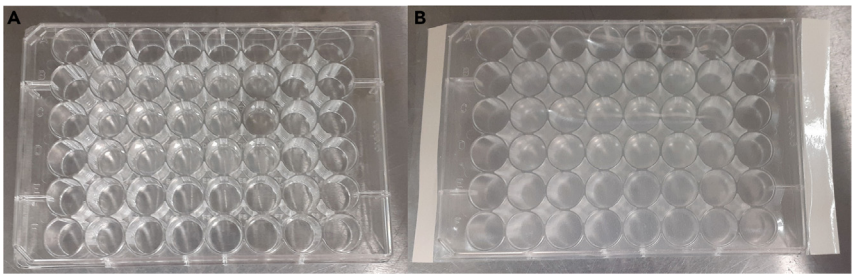

Figure 6. R. palustris fluorescence test preparation

(A) Example of a prepared expression plate. Individual wells are prepared in triplicate with 500 μL of inoculated *R. palustris* media containing transformed strains of *R. palustris*, as well as a triplicate set of Wild Type *R. palustris*.

(B) An expression plate covered with a Breathe-Easy® Permeable Membrane, which provides aerobic conditions while maintaining monoculture conditions.

as the optimal absorption wavelength of the bacteria of interest in the settings of the accompanying program.

k. Export expression data to Microsoft Excel or other appropriate data analysis software. Determine expression fold change values for each culture.

Note: Expression Fold Change can be calculated using the following equation:

$$EFC = \left(\frac{\frac{(Fluorescence - MediaF)}{(Absorbance - MediaA)}}{\left(\frac{(WTFluorescence - MediaF)}{(WTAbsorbance - BlankA)} \right) Avg} \right) Avg$$

Where,

EFC = Expression Fold Change;
Fluorescence = Fluorescence of transformed bacterial culture;
MediaF = Fluorescence of media blank;
Absorbance = Absorbance of transformed bacterial culture;
MediaA = Absorption of media blank;
WTFluorescence = Fluorescence of Wild Type control culture;
WTAbsorbance – Absorbance of Wild Type control culture.

l. Determine significance between transformed cultures and wild-type cultures by performing student's two tailed t-test assuming unequal variances between transformed culture and Wild Type culture EFC values.

Optional: The strength of various terminator sequences can also be determined using this protocol. Termination of transcription does not necessarily end at the stop codon, and so various terminator sequences can be used as controls for terminator regulation. Placing a fluorescent marker on the same strand in series with an antibiotic resistance gene, such as in Figure 7, can allow for an intergenic region to test terminators. Depending on the strength of the terminator sequence, the fluorescence level of cultures transformed with these plasmids.

Determining expression level through RT-qPCR

Timing: 3 days

These steps in the protocol describe a method by which RNA is extracted from *R. palustris* and is subsequently analyzed for expression levels using RT-qPCR. This procedure follows very closely

Figure 7. Depiction of a plasmid construct with a terminator between an antibiotic resistance gene and a fluorescent protein

The intergenic region allows for the insertion of various terminator sequences to control the amount of transcriptional readthrough that occurs from the antibiotic resistance gene. Various terminator sequences can be tested with the Fluorescence Marker Expression Test of this protocol.

from the TRIzol™ extraction procedure, which can be found at https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf, with some exceptions. The RNAlater procedure is also used to store samples for later RNA extractions in this protocol. Other methods of preserving cell cultures for later RNA extraction may also be used.

10. RNA extraction sample preparation (1 day).
    a. Harvest cultures upon reaching the mid-exponential phase by transferring to a 50 mL conical tube.
    b. Samples may be saved at this point for extraction at a later date using the RNAlater protocol found at https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2F7020M.pdf.
        i. Centrifuge cultures at 3,000 × *g* for 15 min at 4°C.
        ii. Resuspend cultures with 50 mL ice-cold PBS.
        iii. Centrifuge at 3,000 × *g* for 15 min at 4°C.
        iv. Repeat previous two steps.
        v. Resuspend cell cultures in 5–10 volumes of RNAlater solution.

    Note: *R. palustris* cultures are typically reduced to 1–2 mL of cell pellets at this point before addition of RNAlater, although differing OD_{660} before centrifugation may change the final volume of cell culture.

        vi. Incubate cell cultures for 24 h in a 4°C room.
        vii. Add equal volume ice cold PM or 1 × PBS and centrifuge cultures at 3,000 × *g* for 15 min at 4°C. Remove supernatant.

    Note: RNAlater must be removed by addition of another less dense media such as 1 × PBS as the density of RNAlater prevents pelleting of cultures.

        viii. Store at −80°C until extraction.

11. TRIzol™ extraction protocol (5–10 h, dependent on number of samples).

⚠ CRITICAL: Throughout the extraction procedure, RNaseZap™ should be applied to all tools that are not already specified as RNase-free and all working area surfaces to prevent contamination of RNase into samples. Tools that interact with surfaces that have not been applied with RNase Zap should be reapplied with RNaseZap™. Any contamination of RNase can cause RNA degradation and destroy the usability of samples for subsequent qPCR. A lab coat, appropriate lab gloves (neoprene gloves are suitable), and safety glasses should be worn throughout the procedure.

    a. Starting from either RNAlater prepped samples or extracting immediately from recently harvested samples, add 1 mL of TRIzol™ reagent to samples in a fume hood and transfer to a 2 mL RNase-free Eppendorf tube.
        i. Pipette up and down to thoroughly mix and vortex each sample for 10 s each.

b. Incubate on ice for 5 min.
c. Add 200 μL chloroform.
d. Cap samples and shake by hand for 15 s.
e. Incubate at 21°C for 5 min.
f. Centrifuge at 12,000 × *g* for 15 min at 4°C.

Note: At this point, the sample should separate into three phases, the lower TRIzol-Chloroform layer, an intermediate layer that contains *R. palustris* DNA, and the upper aqueous layer which contains the RNA.

g. Extract approximately 400 μL of the aqueous phase, making sure to not draw out either the interphase or the phenol-chloroform phase.

Note: It may be advisable to leave 50–100 μL of the aqueous phase with the rest of the sample in order to not disturb either the interphase or the lower phenol-chloroform phase.

h. Add an equal volume of pure Isopropanol and mix by inverting tubes 20 times.
i. Incubate at 21°C temperature for 10 min.
j. Centrifuge samples at 12,000 × *g* for 30 min at 4°C.

Note: *R. palustris* RNA forms a white pellet as opposed to a transparent pellet for *E. coli* as depicted in Figure 8. Figure 8A shows an RNA extract sample before centrifugation with Isopropanol, while Figure 8B depicts the same sample after centrifugation, where RNA from *R. palustris* cultures have precipitated into a white pellet.

k. Remove supernatant, making sure to not draw out the RNA pellet.
l. Resuspend the RNA pellet in 1 mL of ice-cold 75% ethanol and vortex the sample briefly.
m. Centrifuge at 7,500 × *g* for 10 min at 4°C and remove supernatant.
n. Air dry the pellet for 5–10 min or until all of the ethanol has evaporated.

Note: The RNA should be dried completely, but not overdried as the RNA will lose solubility.

o. Resuspend the RNA pellet in 44 μL of RNase-free water and mix by pipetting up and down.

Note: This volume allows for easier calculations of volumes required for subsequent DNA treatment and also provides a sufficient concentration of RNA for reverse transcription to cDNA and qPCR.

p. Incubate at 50°C for 15 min.
q. Remove samples to ice and measure concentration using a microspectrophotometer.
r. Store RNA extracts at −80°C until DNase treatment.

Pause point: Prepared RNA samples are stable at −80°C for 6–12 months.

s. Perform a DNase treatment on each sample to remove any DNA contamination from the RNA extraction process.

⚠ CRITICAL: Immediately store samples at −80°C when not in use.

Note: Depending on the amount of DNA contamination, the DNase treatment may have to be repeated to effectively remove all DNA contamination from samples.

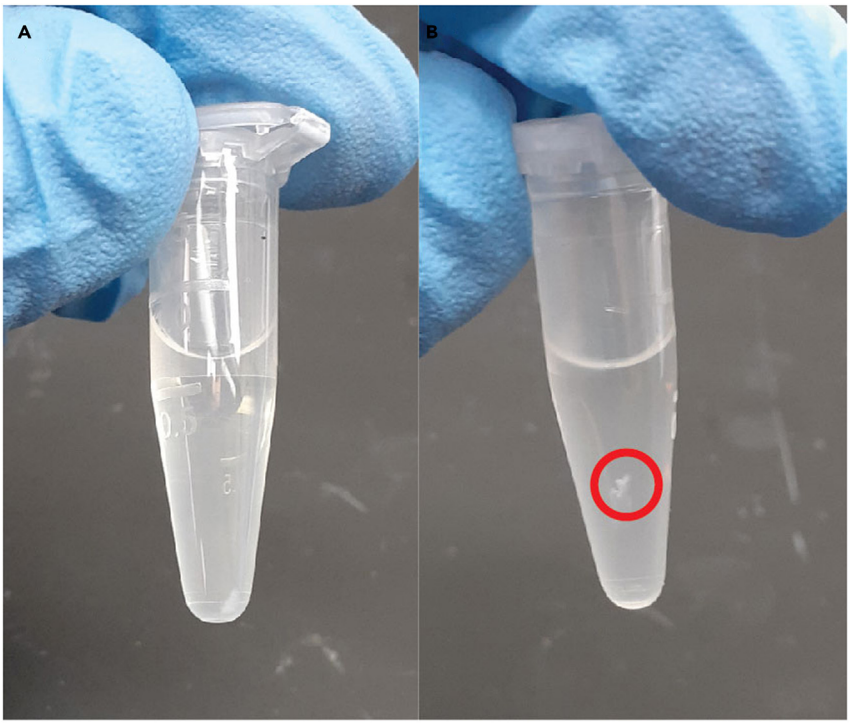

Figure 8. RNA extraction of *R. palustris*

(A) 100% Isopropanol is added to aqueous layer extracts from the TRIzol™ extraction process.

(B) RNA pellet from *R. palustris* samples is precipitated through centrifugation. RNA from *R. palustris* samples appeared as a white pellet.

t. Perform a PCR test of the DNase treated RNA samples with any set of primers that produces a correct band on a DNA template, but no band without a DNA template.

Note: If samples produce a correct band on a 2% agarose gel, the DNase treatment (s) must be reperformed.

u. Measure sample RNA concentration with a microspectrophotometer and record to prevent future RNA losses, immediately store at −80°C.

12. RNA Degradation Check (2–3 h).

Note: This procedure is used as a more accessible method which utilizes cheap common biology lab materials such as bleach, agarose gels, and DNA stains to determine degradation of RNA. This protocol is largely adapted from Aranda et al.[9]

a. Prepare a bleach gel mixture to final concentrations of 1% agarose (w/v) and 5% or 6% any commercialized bleach (v/v) to a TAE buffer (other concentrations of bleach can be used as long as final concentrations are kept).
b. Heat mixture to near boiling for 90 s, let cool until it reaches 21°C.
c. Add 15 μL of SYBR Safe DNA Gel Stain.

⚠ CRITICAL: Ensure that the stain is added just before the solution starts to solidify. The bleach combined with the raised temperature degrades the stain prematurely. The bleach

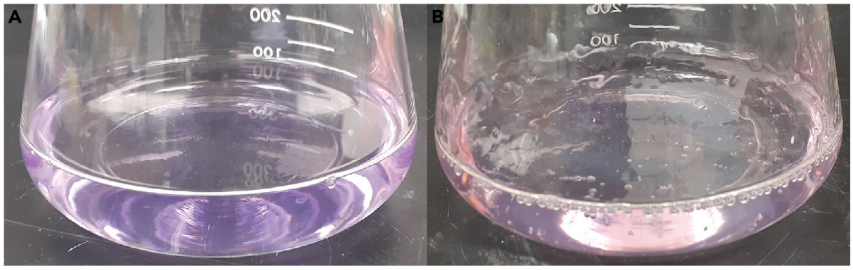

Figure 9. Addition of DNA stain to a bleach gel

(A) An example where DNA stain was added while the 1× TAE agarose solution was too hot, and the gel turns a dark purple. Exposure to bleach at high temperatures appears to destroy the DNA stain. SYBR™ Safe DNA Gel Stain is used in this case.

(B) An example of a viable gel mixture. A red hue colors the mixture as with other gels using SYBR™ Safe DNA Gel Stain.

gel will turn dark purple if the stain is added when the solution is too hot. See Figure 9 for reference on a viable bleach gel.

d. Pour the mixture into an electrophoresis gel mold.
e. Perform gel electrophoresis.

Note: Electrophoresis with the bleach gel can be hotter than electrophoresis with standard gels. Care should be taken to ensure that gel trays do not overheat during the electrophoresis. As a result, lower voltages may be chosen to avoid this problem.

f. Visualize Gel using SYBR Safe stain gel visualization protocol.
   i. All RNA samples that produce two equally bright bands as seen in well 3 of Figure 10, can be used for subsequent qPCR (troubleshooting 4).

Note: If the RNA degradation has been sufficiently prevented, each sample should produce two bands as seen in Figure 10.

g. Convert RNA samples to cDNA using a reverse transcriptase, following the reverse transcription protocol.

Pause point: cDNA samples are stable at −20°C for up to one year.

13. Primer Efficiency (up to 4 h).

Note: Primer efficiency is determined by preparing qPCR reactions with PowerUp™ SYBR™ Green Master Mix, or a similar binding dye, with a 5× dilution series of cDNA consisting of 8 samples ($3.2*10^{-2}$ - 500 ng/µL).

a. Make a set of master mix reactions as described below.

⚠ CRITICAL: When preparing any RT-qPCR, it is essential that reactions are protected from outside DNA contamination as any contaminant may drastically change C_t values of RT-qPCR reactions. A separate set of negative control reactions can be prepared by adding master mix to a well, and instead of cDNA, DNA free water should be added. These reactions should not fluoresce at all during qPCR.

b. Using a real time thermal cycler, prepare reaction settings for the binding dye used.
c. Load reactions into the real-time thermocycler and perform PCR.

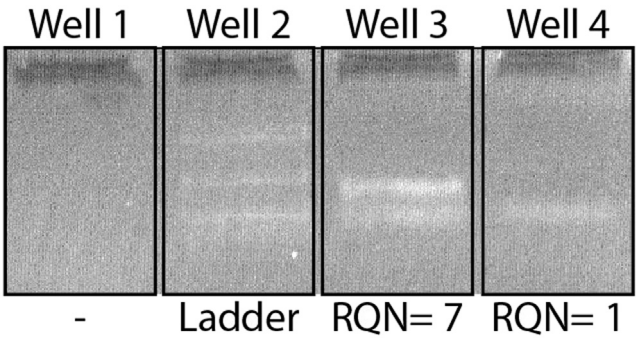

Figure 10. Bleach gel with RNA samples

Well 1 is a negative control, well 2 contains DNA ladder. Well 3 contains an RNA sample with an RNQ (RNA Quality Number) of 7, which is sufficient for future qPCR. Well 4 contains a nearly completely degraded sample of RNA with an RNQ number of 1.

d. Export C_T values as csv or equivalent data file for performing primer efficiency analysis.
e. Plot C_t values vs $\log_{10}$(DNA copies) for each primer reaction set, perform linear regression analysis, and use the ThermoFisher Scientific qPCR calculator to determine the primer efficiency for each primer reaction set based on the slope of the line as in Figure 11.

Note: The calculator can be found at https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html.

Note: Primer reaction sets that have efficiencies between 90% and 110% can be used to obtain C_T values for qPCR reactions for cDNA samples.

f. Primer concentrations with verified primer efficiencies may now be used for subsequent qPCR. All Ct values obtained from qPCR reactions should be within the range of 90%–110% efficiency.
g. Perform qPCR for genes of interest and analyze gene expression levels using $\Delta\Delta C_T$, quantitative transcriptomics, or other methods of determining expression level.

14. Determining Plasmid Copy Number Through qPCR (6 h).

Note: qPCR with gDNA can be performed to determine the copy number of both native and non-native plasmids relative to the chromosome. Most bacteria usually contain one chromosome, but some may be polyploids, where a cell can contain more than one copy of its chromosome. This protocol allows for the indirect measure of stability of non-endogenous plasmids employed in *R. palustris*, and can feasibly be used in other bacteria. This protocol was largely adapted from Delorenzo et al.[10]

a. Incubate *R. palustris* cultures at 30°C and 275 rpm until cultures reach mid-exponential phase.
b. Extract gDNA from cultures using a gDNA extraction kit that extracts both chromosomal and plasmid DNA.

Pause point: gDNA and pDNA extracts are stable at −20°C for several years.

c. Design a set of qPCR primers for a sequence that appears only once in the chromosome, and another set for a sequence that appears only once in the plasmid (ideally the origin of replication).

Note: These primers should also be verified to amplify only the desired region using the Evaluation of PCR Primers section found in Strain handling and construction.

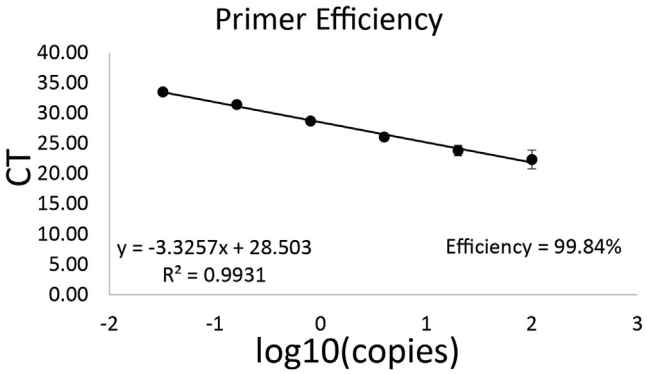

Figure 11. Ct values plotted against log10 of cDNA concentration for one primer set

The slope of the line is used to determine the primer efficiency of the primer set using the Thermofisher qPCR Efficiency Calculator. R^2 values should ideally be above .98 for confirming primer sets. Error bars represent one standard deviation for n = 3.

d. Perform GoTaq® PCR of two targeted genes using primers designed for qPCR.
e. Verify that the PCR product contains only the desired amplicon through gel electrophoresis.
f. Purify the remaining PCR product and measure with a microspectrophotometer.
g. Using the following equation, prepare an external standard curve of purified PCR product using the following equation. Initially dilute amplified PCR product to 10^9.

$$Amplicon\ copy\ number\ =\ \frac{\left(6.02 * 10^{23}\frac{copies}{mol}\right)\left(gDNA\frac{ng}{\mu L}\right)}{(amplicon\ legnth\ (bp))\left(660\frac{g}{mol*bp}\right)}$$

h. Prepare an external standard curve by making a 5× dilution series initially diluting samples to 10^7 copies/µL in triplicate.
i. Perform qPCR of both the dilution series and gDNA extracts of cultures harvested at mid-exponential.
j. Calculate copy number of both the plasmid amplicon and chromosome amplicon by referring to the C_T values obtained from the dilution series external standard curve.
k. Calculate plasmid copy number using the following equation.

$$Plasmid\ copy\ number\ ratio\ =\ \frac{Copies\ of\ gene\ from\ plasmid}{Copies\ of\ gene}$$

GoTaq® PCR Master Mix (DNA Template)	
Reagent	Amount
DNA template	100 ng
GoTaq® Green Master Mix 2×	12.5 µL
Primer 1	50–350 nM
Primer 2	50–350 nM
ddH_2O	Balance to 25 µL

GoTaq® PCR Master Mix (No DNA Template)	
Reagent	Amount
DNA template	-
GoTaq® Green Master Mix 2×	12.5 µL
Primer 1	50–350 nM
Primer 2	50–350 nM
ddH_2O	Balance to 25 µL

PowerUp™ SYBR™ Green Master Mix (Primer Efficiency)	
Reagent	Amount
DNA template	(0.0061 ng/μL – 500 ng/L) OR previously determined from primer efficiency tests
PowerUp™ SYBR™ Green Master Mix	10 μL
Primer 1	Determined by initial evaluation
Primer 2	Determined by initial evaluation
ddH_2O	Balance to 20 μL

GoTaq® PCR			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	120 s	1
Denaturation	95°C	45 s	40 cycles
Annealing	60°C	45 s	
Extension	72°C	30 s per 1,000 bp	
Final extension	72°C	5 min	1
Hold	4°C	forever	

PowerUp™ SYBR™ Green PCR			
Steps	Temperature	Time	Cycles
Initial Denaturation	50°C	120 s	1
Denaturation	95°C	120 s	40 cycles
Annealing	95°C	15 s	
Melting Curve	95°C	15 s	1
	60°C	15 s	1
	1.5°C/min	20 min	1
	95°C	15 s	1
Hold	4°C	forever	

EXPECTED OUTCOMES

This protocol aims to provide a method for constructing a synthetic biology toolkit for *R. palustris* and other non-model bacteria in general. The toolkit constructed in this protocol includes a method of incorporating heterologous genes in non-model bacteria and measuring their expression level using fluorescent reporters, as well as a method for tuning expression using differing RBS sequences. Gene expression from *R. palustris* specifically can alternatively be determined directly using the RT-qPCR method provided in this protocol. As RNA from *R. palustris* appears to be more difficult to extract than RNA from other organisms, a modified method of the TRIzol™ extraction protocol has been provided.

Figure 12 shows a summary of the synthetic biology "parts" to be characterized by this protocol. Non-model organisms can be more recalcitrant to transformations by incorporating non-endogenous plasmids than model organisms such as *E. coli*. It therefore can be advantageous to possess a set of synthetic biology parts that have been characterized to reliably express heterologous genes of interest. Parts that are known to regulate expression level such as RBS sequences can then be used in future expression systems to employ genes from metabolic pathways these non-model organisms do not possess. One of the methods bacteria employ to ensure that plasmids are maintained across generations is to replicate plasmids to have a high enough copy number so that the possibility a daughter cell will not possess a plasmid is insignificant. However, the maintenance of many copies of a single non-endogenous plasmid can be metabolically taxing. Moreover, expression of genes of interest into the genome of *R. palustris* can be more reliable than plasmid-based expression as non-endogenous plasmids can be lost over generations. Therefore, a second method of incorporating heterologous genes into the genome of *R. palustris* has also been provided by this protocol using double-homologous recombination.

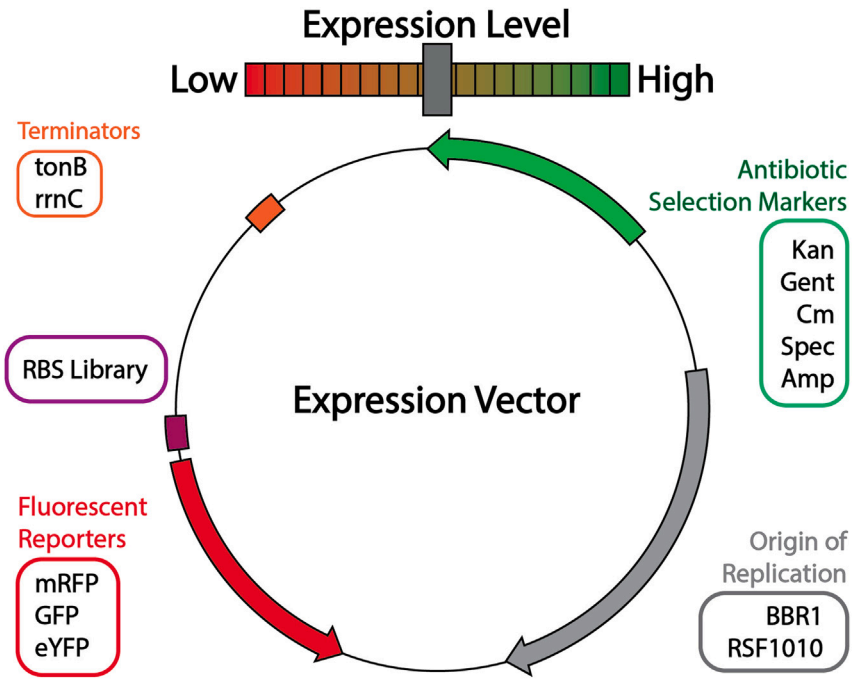

Figure 12. Graphical summary of the synthetic biology toolkit that this protocol aims to assemble in non-model bacteria

Various synthetic biology parts are optimized to provide a vector for measuring heterologous gene expression, as well as methods to tune expression to desired levels.

LIMITATIONS

The steps in this protocol have been specifically optimized for *R. palustris* and may not perform as effectively in other non-model bacteria. This protocol is meant to be used as a guide for constructing synthetic biology toolkits in other non-model bacteria. The sections for Evaluation of PCR Primers, RBS Library Construction, Observing Plasmid Stability via Fluorescent Markers and Flow Cytometry, Fluorescent Marker Expression Test, Determining Plasmid Copy Number Through qPCR, can be applied to other non-model bacteria without need for optimization, though incubation conditions and growth times may need to be adjusted to account for the various growth rates of other bacteria. Other sections such as the Strain Construction, Transformation by Electroporation, or RNA Extraction may need to be optimized for other organisms as tools for editing the genetic content of non-model organisms may not work as well across species.

TROUBLESHOOTING

Problem 1

Reactions containing no DNA produce bands of the correct size during Evaluation of PCR Primers in strain handling and construction.

Potential solution

The phenomenon where the correct band length is produced from reactions without any DNA template has not been fully investigated. New stocks of water, primers, and Gotaq® Master Mix can be used to verify a lack of contamination of DNA template. If the problem persists, a redesign of primers may be advisable.

Problem 2

Wells in expression tests have experienced severe evaporation in fluorescent marker expression test.

Potential solution

Wells that undergo severe evaporation can lead to inaccuracies in measuring fluorescence intensities as the concentration of cells is being artificially raised. To prevent evaporation, it may be advisable to provide a source of humidity in the incubator being used to grow cultures. Another method to prevent evaporation is to not use the wells that are on the perimeter of the 48 Well Suspension Culture Plate as these wells typically experience more evaporation.

Problem 3

Plasmids appear to not be retained during incubation, or fluorescence intensity drops over time during Fluorescent marker expression test.

Potential solution

As discussed in Immethun & Kathol et al.,[1] it appears that some combinations of antibiotic selection and origins of replication are not compatible with *R. palustris*. A different antibiotic selection marker or origin of replication may need to be used as a result.

Problem 4

The top band of RNA samples does not appear in a bleach gel test as seen in Figure 10 during RNA Degradation Check in determining expression level through RT-qPCR.

Potential solution

RNA degradation has not been sufficiently prevented. This can result from a lack of applying RNaseZap™ to tools that come into contact with RNA extraction samples or from a lack of high RNA concentration. To increase the amount and purity of RNA extracted from samples, it may be advisable to divide the RNA extractions after the addition of TRIzol into 2 separate samples. This can be done at step 11a in the "determining expression level through RT-qPCR" section by adding an additional 1 mL of TRIzol and pipetting half of the solution to a separate RNase-free Eppendorf tube. Both samples should be treated independently and processed identically using the above protocol until recombining at step 11o, where 22 μL of RNAse-free water should be added to the halved samples instead of 44 μL, and combined into a single Eppendorf tube.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rajib Saha, rsaha2@unl.edu.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

M.K. and R.S. formulated the idea for this work. C.I.developed the methodology. C.I. and M.K. constructed strains and conducted tests. All authors analyzed the data. M.K. wrote the manuscript with guidance from C.I. and R.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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