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Genetic Tools Towards A Synthetic Biology Approach For Whole Mitochondrial Genome Engineering

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Abstract

Synthetic biology is an interdisciplinary research field that standardizes and repurposes biological components to better understand life, solve complex problems and produce superlative organisms for industry. As synthetic biology has developed, the goal has become to generate fully controllable systems through whole genome engineering (WGE), the cumulation of standardized genome engineering protocols, and DNA delivery methods. In eukaryotes, genetic tools for WGE are limited to the nucleus and present a need to expand to include mitochondria, which maintain their own unique genome and produce energy for the cell. The work presented here begins developing the resources needed to enable whole mitochondrial genome engineering.

First, to standardize mitochondrial genome engineering protocols, I cloned the mitochondrial genomes of two diatomaceous algae, *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*, as plasmids in *Escherichia coli* and *Saccharomyces cerevisiae* using PCR-based and transformation-associated recombination cloning methods. Next, a PCR-based engineering method was optimized to generate derivative algal mitochondrial genomes cheaper than the cost of DNA synthesis in approximately ten days. Additionally, minimal host burden and plasmid instability was detected for the mitochondrial plasmids making them suitable cargo for delivery experiments.

After, I sought to adapt an entirely *in vivo* DNA delivery method, bacterial conjugation, for mitochondrial transformation. Two approaches have been envisioned for bacterial conjugation to mitochondria, cell fusion of a bacteria containing a mitochondrial plasmid followed by intra-cellular bacterial conjugation, and engineering key conjugative proteins with the addition of a mitochondrial localization signal. In either scenario, DNA transfer rates will likely decrease beyond the rate of detection. Therefore, I first looked to improve DNA transfer to eukaryotes by generating and screening a deletion plasmid library for the broad-host range conjugative plasmid, pTA-Mob 2.0. From this data, pSC5 was created that improved DNA delivery to yeast. The utility of pSC5's superior DNA delivery was demonstrated by creating the pSC5-toxic plasmids, which contained genes toxic or partially toxic to yeast. The pSC5-toxic plasmids effectively killed yeast and established a

novel first-in-the-world conjugation-based antifungal. Together these resources for mitochondrial genome engineering and improved DNA delivery should improve the feasibility of future endeavors in whole mitochondrial genome engineering.

Keywords: *Phaeodactylum tricornutum*, *Thalassiosira pseudonana*, *Metschnikowia*, *Candida*, yeast, algae, bacterial conjugation, antifungal, mitochondrial plasmid, mitochondrial genome engineering

Lay Summary

Cells can be viewed as microscopic machines, such as a computer. Like a software program, their DNA encodes instructions, which the operating system interprets as commands based on various inputs, similar to a cell sensing its environment. A cell's complete set of instructions is referred to as a genome, and scientists can modify genomes to alter how and what the cell does, including producing medicines or storing carbon, for instance. However, with current technologies in most cells, typically, only a few edits to a genome can be made at a time, making the process tedious and labor-intensive.

Recently, technologies that enable researchers to modify entire genomes simultaneously have become available. These technologies are referred to in their entirety as whole genome engineering (WGE). WGE is accomplished by writing a new desired genome and building it from scratch using chemical methods called DNA synthesis. The synthetic genome's instructions are verified and then delivered into a cell lacking DNA. These technologies have been developed for relatively simple bacteria; however, more complex cells lack WGE methods and can each contain 1–3 different genomes that could be modified. The main limiting factors are an absence of standardized methods for modifying entire genomes of more complex cell types and difficulties delivering them where they need to go.

This thesis develops foundational technologies required to enable future WGE of the genome located within a cellular compartment called the mitochondria, widely known for its role in energy production. Specifically, I developed standardized methods for modifying mitochondrial genomes in approximately ten days at a fraction of the cost of DNA synthesis. After, I sought to improve DNA delivery to complex cells using a method called bacterial conjugation. I improved this DNA delivery method to fungi 23-fold and showed it could be used as an antifungal. Future research should attempt to adapt this DNA delivery method's specificity in complex cells, from indiscriminate DNA delivery anywhere inside the cell to specifically their mitochondrial compartment. Now, WGE of mitochondria requires developing innovative ways to identify what cells have successfully received a genome and deciding what to make them do.

Co-Authorship Statement

For the work presented in Chapters 2–4, R. R. Cochrane and B. J. Karas conceived the experiments. R. R. Cochrane performed the experiments, and R. R. Cochrane and B. J. Karas analyzed the results. R. R. Cochrane created the figures and wrote the manuscripts with input from B. J. Karas. Exceptions and additional contributions for each Chapter are noted below.

In regard to Chapter 2:

S. Hamadache performed analysis of sequencing data used in Table 2-2 and Supplementary Table B-2, and created plasmid maps used in Figure 2-2A). M. P. M. Soltysiak assembled and screened the plasmids pPT-PCR C2-1 and C2-2 used in Figure 2-2 C, D), and Tables 2-1 and 2-2. S. L. Brumwell performed growth experiments, analyzed data, and created figures for Figure 2-3C, D). P. Janakirama of Designer Microbes Inc. assembled the plasmids pPT-PCR C1 and C2.

In regard to Chapter 3:

S. Hamadache performed analysis of sequencing data and generated Table 3-2 and Supplementary Table C-2, and created plasmid maps used in Figures 3-1A, B), 3-4 A, C), and Supplementary Figure C-2. S. L. Brumwell performed growth experiments, analyzed data, and created Figure 3-2. A. Shrestha analyzed data and created Figure 3-3, and performed RNA extraction for Supplementary Tables C-3 and C-4. D. J. Giguere generated and analyzed the RNA sequencing data in Supplementary Tables C-3 and C-4.

In regard to Chapter 4:

R. S. Shapiro contributed to conception of experiments involving *Candida* species and assisted in the writing of the manuscript. M. A. Lachance provided yeast strains listed in Section 4.4.2. B. J. Karas created the graphics of Figures 4-1, 4-3, 4-4A), 4-6A), 4-7A). S. L. Brumwell created the plasmids pAGE1.0, pAGE2.0.T, and pAGE2.0-i listed in Table 4-1. H. H. Say sequenced all previously unpublished plasmids listed in Table 4-1. A. Shrestha performed, analyzed data, and created Figures 4-2, 4-4 B) (Note: for *E. coli*, data analysis only), 4-5, and 4-7, and Supplementary Figures D-3, D-4, D-6, and D-7; and created Supplementary Tables D-2, D-5, D-6, and D-10. C. Tong assisted in the generation of data

for Figures 4-2, 4-4B), 4-5B, C), and 4-7, Supplementary Figures D-6 and D-7, and Supplementary Tables D-3, D-5, D-6, and D-10. K. Van Belois assisted in the generation of data for Figures 4-2 and 4-7, and Supplementary Table D-3. E. J. L. Walker generated the data for Figure 4-3, and designed, built, and screened pSC5-toxic2. J. S. Meaney performed *E. coli*-to-*E.coli* bacterial conjugation for Figure 4-4B), and Supplementary Table D-5. M. Agyare-Tabbi performed and analyzed data pertaining to the bacterial conjugation of plasmids to *Candida* species and created figures for Figure 4-5A), and Supplementary Figure D-4. D. P. Nucifora designed, built, and screened pSC5-toxic1. M. M. S. De Almeida created and screened many plasmids of Supplemental Table D-3. S. Hamadache performed analysis of sequencing data M3C1 and M3C2 only, and generated Supplementary Table D-4.

Dedication

This thesis is dedicated to my parents Joseph Bruce Cochrane and Gail Allen whose logical worldview, relentless work ethic and self-sacrifice have offered me every opportunity to succeed in life. You have cultivated an unshakeable self-confidence in my mind and heart that anything is achievable with discipline and kindness. Thank you.

"Quit, don't quit… Noodles, don't noodles… You are too concerned with what was and what will be. There is a saying: Yesterday is history, tomorrow is a mystery, but **today is a gift**. That is why it is called the present."

— Grand Master Oogway

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List of Abbreviations and Symbols

Ω	ohm
~	approximately
<	lesser than
>	to
+	plus
±	plus-minus
=	equals
%	percent
°C	degrees Celsius
-HIS	synthetic complete media lacking histidine
-TRP	synthetic complete media lacking tryptophan
α	alpha
β	beta
μE	microeinstein
μF	microfarad
μg	microgram
μL	microliter
μM	micromolar
aacC1	aminoglycoside-(3)-*N*-acetyltransferase
ACT1	yeast actin
ade2	phosphoribosylaminoimidazole carboxylase
Ala	alanine
aphA	aminoglycoside 3'-phosphotransferase
Arg	arginine
ARG8$^{\mathrm{m}}$	acetylornithine aminotransferase encoded with translation table 3
ARSH4	autonomous replication sequence
Asn	asparagine
Asp	aspartic acid
AT	adenine and thymine
ATCC	American Type Culture Collection
atp	mitochondrial ATP synthase (Complex V)
bla	beta-lactamase
bp	base pair
C	clone
Ca	*Candida auris*
Cas9	CRISPR-associated protein 9
cat	chloramphenicol acetyltransferase
Cbe	*Candida* aff. *bentonensis*
Cbr	*Candida bromeliacearum*
CCAP	Culture Collection of Algae and Protozoa
CCIB	Computational and Integrative Biology
cDNA	complementary DNA
CEN6	*Saccharomyces cerevisiae* chromosome 6 centromere
CFU	colony forming unit

cir^0	lacking 2μ plasmid
CM	chloramphenicol
cob	mitochondrial cytochrome b (Complex III)
cox	mitochondrial cytochrome c oxidase (Complex IV)
CRISPR	clustered regularly interspaced palindromic repeats
Ct	*Candida tolerans*
Cub	*Candida ubatubensis*
cysG	siroheme synthase
D	directly
DNA	deoxyribonucleic acid
d-*nat*	*N*-acetyltransferase encoded for diatoms
dum	dark uniparental minus
EDTA	ethylenediaminetetraacetic acid
F	forward
fcpD	fucoxanthin chlorophyll a/c binding protein D
fiw	fertility inhibition of incW plasmids gene
fmol	femtomole
FUM^m	fumarase protein encoded with translation table 3
g	gram
G0	generation zero
G60	generation sixty
G+C	guanine plus cytosine
gDNA	genomic DNA
GFP	green fluorescent protein
GFP^m	green fluorescent protein encoded with translation table 3
GG	Golden Gate
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GXL	**G**C-rich templates, e**x**cess template, and **l**ong amplicons
h	hour
HCA	*HIS3-CEN6-ARSH4*
HF	high fidelity
His	histidine
HIS3	imidazoleglycerol-phosphate dehydratase
I	induced
ID	indirectly
Ile	isoleucine
Inc.	incorporated
IQR	interquartile range
IR	inverted repeat
ist	insertion sequence transposition function gene
kbp	kilobase pairs
kfr	*korF*-regulated function gene
Kil	host-killing function when depressed gene
kla	KilA phenotype gene
kle	KilE phenotype gene
kor	host killing override/gene regulation gene

kV	kilovolt
L	liter
L1	synthetic seawater
LB	lysogeny broth
LBmc	lysogeny broth supplemented with magnesium sulfate and calcium chloride
Leu	leucine
Lys	lysine
lys2	L-2-aminoadipate reductase
m	meter
M	molar
MATα	mating type alpha
Mb	*Metschnikowia borealis*
Mbp	megabase pairs
Met	methionine
met14	adenylyl-sulfate kinase
mg	milligram
Mg	*Metschnikowia gruessi*
min	minute
mL	milliliter
Ml	*Metschnikowia lunata*
mm	millimeter
mM	millimolar
Mp	*Metschnikowia pulcherrima*
MPX	multiplex
mrfp	monomeric Red Fluorescent Protein
mRNA	messenger RNA
Mt	mitochondrial
n	sample size
N	nourseothricin resistance gene
nad	mitochondrial NADH dehydrogenase (Complex I)
nat	*N*-acetyltransferase gene
NEB	New England Biolabs
ng	nanogram
OD	optical density
ORF	open reading frame
oriT	origin of transfer
p	p-value
P	primer
par	plasmid partitioning gene
PCR	polymerase chain reaction
pDNA	plasmid DNA
pH	potential of hydrogen
Phe	phenylalanine
PMSF	phenylmethylsulfonyl fluoride
Pro	proline
pSC5	pSuperCon5
psi	essential translation termination factor protein – Sup35p
R	reverse

R⁻	restriction minus
RCF	relative centrifugal force
rDNA	recombinant DNA
RecA	recombinase
rep	origin of replication
Rep	replicate
rho⁰	absent mitochondrial DNA
rho⁻	mutated mitochondrial DNA
*RIP1*ᵐ	Rieske FeS protein encoded with translation table 3
RNA	ribonucleic acid
rpl	mitochondrial ribosomal protein for large subunit
RPM	revolutions per minute
rps	mitochondrial ribosomal protein for small subunit
rRNA	ribosomal RNA
rrnL	genes for large subunit of ribosomal RNA
rrnS	genes for small subunit of ribosomal RNA
rrsA	16S ribosomal RNA
RT	room temperature
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
s	second
Sc	*Saccharomyces cerevisiae*
sddH₂O	sterile double-distilled water
Ser	serine
Sh ble	bleomycin resistance protein from *Streptoalloteichus hindustanus*
SOC	super optimal broth with catabolite repression
SPEM	sorbitol-EDTA-sodium phosphate dibasic heptahydrate-sodium phosphate
SSB	single stranded DNA-binding protein
STOP	stop codon
T	thousand units per gram
TAE	tris-acetic acid-EDTA
TAR	transformation-associated recombination
tat	mitochondrial twin-arginine protein translocation system gene
td	doubling time
TE	tris-EDTA
Thr	threonine
tmtc	too many to count
To	*traJ* ORF mutation
Tp	*traJ* promoter mutation
tra	transfer function A gene (Tra1)
Tra1	transfer operon 1 – mobilization proteins
Tra2	transfer operon 2 – mating-pair formation proteins
TraA–G	primase operon
TraC	DNA primase and single-stranded DNA binding protein
TraF	peptidyl transferase
TraG	coupling protein of *tra* and *trb* functions
TraH	relaxosome protein
TraH–J	relaxase operon
TraI	relaxase

TraJ	*oriT* recognizing protein
TraK	*oriT* binding protein
TraK–M	leader operon
trb	transfer function B gene (Tra2)
TrbC	pilin protein
trf	trans-acting replication function gene
trn	tRNA gene
TRP1	N-(5'-phosphoribosyl)anthranilate isomerase
Tyr	tyrosine
UGA	uracil-guanine-adenine
UI	uninduced
upf	unknown plasmid function gene
URA3	orotidine 5-phosphate decarboxylase
V	volt
Val	valine
w/v	weight by volume
WGE	whole genome engineering
x	times
y-*nat*	*N*-acetyltransferase encoded for yeasts
YKO	Yeast Knockout Collection
YPD	yeast extract-peptone-dextrose
YPDA	yeast extract-peptone-dextrose-adenine

Chapter 1

1   Introduction

1.1   Synthetic biology

Synthetic biology is a contemporary field of study that characterizes life by combining multiple scientific disciplines. It is a vast field that standardizes and repurposes biological components such as DNA, RNA, proteins, metabolic processes, and lipid membranes [1–6]. In research, the reconstruction of cellular processes from their component parts highlights the limitations of current knowledge and informs subsequent inquiries. In industry, life engineered with synthetic properties can improve society and solve complex problems. The emergence of synthetic biology followed the development of three enabling technologies: 1) DNA sequencing, 2) DNA synthesis, and 3) DNA editing. It is now possible to combine these technologies to generate novel synthetic DNA molecules (DNA synthesis), introduce them into living organisms (DNA editing), and rapidly confirm their genotypes (DNA sequencing). It is the role of the synthetic biologist to combine these three technologies to engineer lifeforms effectively and efficiently with unique and valuable characteristics.

The power of synthetic biology is its ability to introduce many modifications to a genome simultaneously. As the field of synthetic biology develops, the goal has become to generate fully controllable systems through whole genome engineering (WGE) [7–17]. This has been realized in the prokaryotes *Mycoplasma genitalium*, *Mycoplasma mycoides*, and *Escherichia coli* by the total synthesis and assembly of entire genomes [9,13–15,17] and has begun in eukaryotes by The Synthetic Yeast Genome Project (*Saccharomyces cerevisiae* 2.0) that is building an entirely customizable synthetic version of the yeast genome, which is more stable than the wild-type strains [16,18–20]. The power of these models is apparent and is being applied gradually to other organisms [10–12]. However, while these systems offer total control over the nuclear genome in eukaryotes, the nucleus is only part of the equation. To enable full control and completely

harness the power of eukaryotes for synthetic biology, it will be necessary to apply the same approach of WGE to their organelles.

1.2   Organelles

Organelles are subcellular structures that have specific roles inside eukaryotic cells. Examples of organelles include the ribosome, endoplasmic reticulum, Golgi apparatus, chloroplast, and mitochondria. Compartmentalization within membrane-bound organelles enables specialized metabolisms within the cell, like complex coordination of energy production and storage. The mitochondria and chloroplast are unique organelles arising from an endosymbiotic relationship resulting in the integration of a bacterium into a host cell that persisted and became specialized.

1.2.1   Endosymbiont theory – Origin of the mitochondria

Symbiosis is a mutually beneficial relationship between two organisms that live in a close physical association. Endosymbiosis is a unique form of symbiosis where one of the symbiotic organisms resides within the other. Endosymbiont theory asserts that the emergence of mitochondria resulted from endosymbiosis and attempts to describe the conditions of eukaryogenesis, the progression of events toward eukaryotic life. There are two contending endosymbiotic models for the emergence of mitochondria: phagotrophy and syntrophy also referred to as mitochondria-late and mitochondria-early models, respectively [21–35].

Briefly, one version of the endosymbiotic theory is the hydrogen hypothesis [27]. It proposes an anaerobic syntrophy between a strictly hydrogen-dependent autotrophic archaebacterium host [36–48] and a heterotrophic facultatively anaerobic bacterium endosymbiont [40,47–55], which are both supported by phylogenetic studies. The symbiosis was progressively strengthened by the extension of the host cell membrane around the bacterium, increasing surface area for the exchange of metabolites and leading

to eventual encapsulation [27,29,31,32,34,36,37,56–58]. Furthermore, the persistence of the intracellular bacterium initiated the conditions for the evolution of the endomembrane system within the archaeal host [24,29,59]. Once internalized, an entire bacterial genome's worth of genes were donated from the endosymbiont to the host genome through endosymbiont gene transfer [55,60–64]. The endosymbiont gene transfer of bacterial genes reinforced the intra-cellular symbiosis [61], replaced many archaeal pathways in the host generating a composite host genome [61,63–65], and resulted in genome reduction of the endosymbiont into the mitochondrial genomes observed today [60,61,64]. The result was a primitive eukaryote with defining traits, including cytoskeleton, separation of transcription and translation spatiotemporally, increased cell size relative to prokaryotes, mosaic linear chromosomes, and, importantly, membrane-bound compartments such as the mitochondria.

1.2.2   The mitochondria

The exact number of mitochondria in a cell differs between species, but in single-celled eukaryotes, there are typically 1–10 mitochondria [66–71]. All mitochondria are composed of an outer and inner membrane. The inner membrane forms infoldings, called cristae, that increase surface area for aerobic respiration and shape the boundary of the mitochondrial matrix where its metabolisms are performed [72–75]. Although mitochondria are commonly represented as bean-like structures, they come in many shapes and sizes and form highly dynamic networks that undergo binary fission and fusion [66,71,72,76].

Mitochondria are widely known as the "powerhouse of the cell" for their integral role in adenosine triphosphate production by oxidative phosphorylation [64,73,77]. However, it also performs many other essential metabolisms for the cell, including pyruvate metabolism, tricarboxylic acid cycle, fatty-acid biosynthesis and β-oxidation, branched-chain amino acid degradation, and the biosynthesis of ubiquinone, biotin, and iron-sulfur clusters [55,73–75,78–84]. Mitochondrial proteomes typically contain 100–1000's of proteins mainly encoded in the nucleus [60,70,81,82]. Mitochondrial proteins

expressed from the nucleus are identified by an N-terminal leader-peptide sequence that signals transport to the mitochondrial translocase of the outer membrane protein [73,85]. The unfolded protein is fed through the mitochondrial translocase of the outer membrane protein and, subsequently, the translocase of the inner membrane protein [73,85], where the leader-peptide sequence is cleaved off, and the protein assumes its mature conformation [73,85]. A small minority of the mitochondrial proteome is encoded in the mitochondrial genome. Mitochondria maintain a core set of genes, including machinery for DNA replication and expression, the electron transport chain, protein import, and protein maturation [55,63,64,86–88]. The mitochondrion usually contains 10–1000 copies of its genome, which is small, compact, and circular [64,71,79,87,89]. However, in some organisms, the genome can be linear [71,79,87,89,90], mini- and maxi-circles [87,91], or completely absent [63,64].

The presence of mitochondrial genomes offers an opportunity for synthetic biologists to engineer eukaryotic life far beyond the capabilities of prokaryotes or the eukaryotic nuclear genome alone. The mitochondria host a diversity of metabolisms with the potential for engineering and offer a sequestered location to express transgenic pathways. Metabolic specialization and compartmentalization of membrane-bound organelles are vital features that need to be exploited to harness the full potential of eukaryotic platforms. However, WGE strategies for mitochondria are required.

1.3   Mitochondrial genome engineering

Genetic manipulations to alter the mitochondrial compartment's function can be targeted to either the nuclear or mitochondrial genomes. Nuclear genome engineering is typically used because of its ease of transformation and extensive genetic toolbox relative to the mitochondria, which lacks genome engineering techniques in many species [92–94]. Native mitochondrial proteins encoded in the nuclear genome are common targets for genetic engineering or the study of mitochondria [95–97]. Alternatively, chimeric proteins can be constructed with transit peptides to localize transgenic proteins or pathways to mitochondria [94,96–102]. However, these methods are limited in the size

and type of proteins that correctly localize within the mitochondria, restricting the diversity of proteins and pathways that can be imported [103–106]. Also, these methods do not enable modifications of the mitochondrial genome directly.

There are several advantages to engineering a mitochondrial genome as opposed to a nuclear genome, including a lack of positional effects [1,6,107,108], RNA interference [6,109], and gene silencing [6,109,110] that are common to the nucleus. The ploidy of mitochondrial genomes increases the copy number of transgenic cassettes resulting in high protein accumulation, and its polycistronic gene organization simplifies recombinant DNA (rDNA) design [87,111]. The mitochondrial compartment enables the localization of synthetic genetic pathways "hidden" from the rest of the cell and the expression of unique proteins containing oxygen-sensitive metal clusters [73,75,112]. Moreover, the expression of protein pathways in the mitochondria can compartmentalize toxic intermediates, increase local concentrations, and minimize competing reactions from the rest of the cell, ultimately increasing product yields [21,96,97,99,100,102,113]. Finally, mitochondrial engineering offers improved control of carbon flux and energetics of eukaryotic cells as the primary site of cellular energy metabolism and regulation [100].

Synthetic biology aspires to facilitate whole mitochondrial genome engineering approaches that enable complete genetic control of eukaryotes. Efficient whole mitochondrial genome engineering requires methods for the rapid generation and testing of derivative mitochondrial genomes with multiple genetic changes. Creation of these methods requires: 1) mitochondrial genomes to be cloned into a host organism for simplified handling and manipulation of DNA, 2) rapid genetic engineering protocols, and 3) robust methods of DNA delivery. Many mitochondrial genomes have been successfully cloned into host organisms; however, robust methods of engineering and transforming whole mitochondrial genomes into mitochondria are lacking.

1.4   Mitochondrial genome cloning

1.4.1   Cloning methods

Molecular cloning is the replication of DNA to produce a population of cells with
identical DNA molecules. It is achieved by assembling rDNA containing vector DNA
(i.e., from the host for DNA maintenance) and source DNA (i.e., from the organism of
interest). The vector DNA minimally includes a selection marker, a promoter and
terminator to drive selection marker expression, and an origin of replication (Figure 1-
1A) [114–120]. For mitochondrial genome engineering, the source DNA consists of a
mitochondrial genome created from either biological (Figure 1-1B) [114,115,119,121–
130] or synthetic DNA (Figure 1-2) [131]. While rDNA can be assembled *in vitro*, it
requires a living host organism to prevent DNA damage by shearing forces, maintain the
cloned DNA, and expand the population of rDNA molecules.

Mitochondrial genomes cloned from biological sources can be captured indirectly
or directly using PCR-based [123–126] or transformation-associated recombination
(TAR) cloning methods [119,120,125], respectively (Figure 1-1B). The vector DNA for
either cloning method is designed with overlapping sequence homology at its termini to
the mitochondrial genome for integration during DNA assembly. In PCR-based cloning,
the source DNA is indirectly amplified in pieces with overlapping sequence homology
[123–126]. Alternatively, in TAR cloning, source DNA is directly isolated from the
organism of interest and linearized using a restriction enzyme with a single cut site
[119,120,125]. In both methods, the rDNA can be assembled either by homology-
directed repair within a host organism or *in vitro* and subsequently transformed into a
host organism. The presence of correctly cloned rDNA is confirmed through *in vitro*
diagnostic tests such as PCR screens, restriction enzyme digestion, and DNA sequencing.
Alternatively, mitochondrial genomes can be cloned *de novo* by DNA synthesis.
Typically, a hierarchical assembly of DNA is used that progressively builds larger
molecules until a complete genome is achieved (Figure 1-2) [12,13,15,16,123,131]. For
example, the mouse mitochondrial genome has been assembled from completely
synthetic DNA oligos [131]. In all cases, the mitochondrial genome must be stored *in*
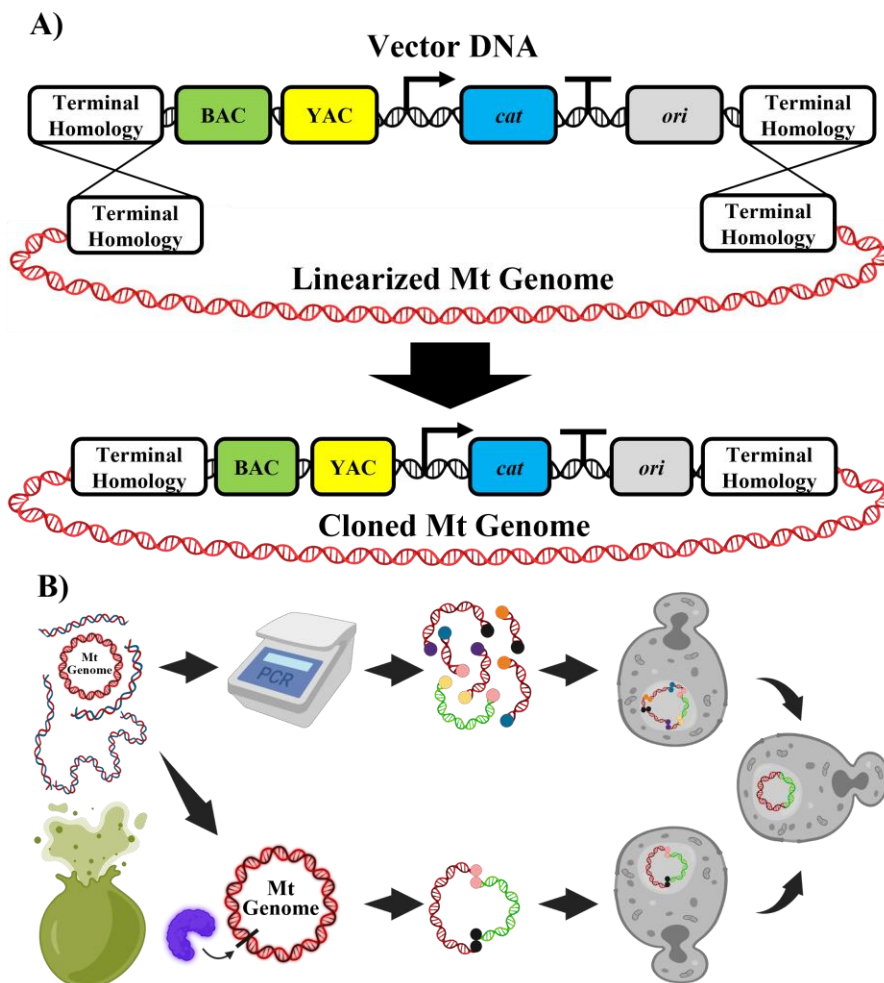
Figure 1-1. Overview of strategies used to clone mitochondrial genomes. A) Schematic of a generic cloning vector (black) containing all the genetic elements required for selection and stable propagation in bacteria (green; BAC – bacterial artificial chromosome), yeast (yellow; YAC – yeast artificial chromosome), and a hypothetical organism of interest (blue and grey; *cat* – chloramphenicol acetyltransferase, and *ori* – origin of replication). At their termini, both the vector DNA and mitochondrial genome (red; Mt – mitochondrial) contain sequence homology (white) to each other. The mitochondrial genome becomes cloned following integration of the vector DNA by homology-directed repair (bottom) and stable maintenance in a host organism. **B)** Schematic of the PCR-based (top) and transformation-associated recombination (bottom; TAR) cloning methods used to clone mitochondrial genomes in yeast. In both methods, total DNA (i.e., nuclear and organellar DNA) is isolated from cells. In PCR-based cloning, the mitochondrial genome (red) and a cloning vector (green) are PCR-amplified with sequence homology to adjacent fragments. In TAR cloning, a mitochondrial genome is linearized by a restriction enzyme (purple) and a cloning vector is PCR-amplified with terminal sequence homology. In both methods, the fragments are pooled and transformed into yeast to generate a cloned mitochondrial genome. Figure created with BioRender.com.
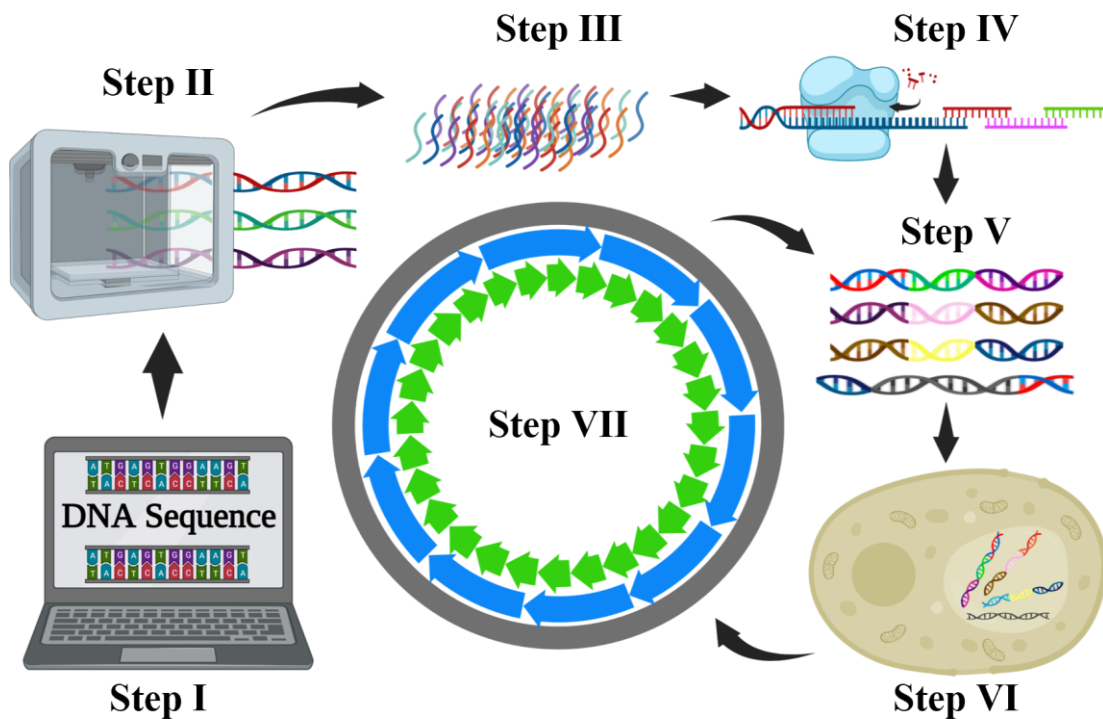
Figure 1-2. Hierarchical strategy for building and cloning mitochondrial genomes *de novo* using synthetic oligonucleotides. A mitochondrial genome is designed and fragmented into short overlapping oligonucleotides *in silico* (Step I), and generated by DNA synthesis (Step II). The short oligonucleotides are pooled (Step III) and joined by PCR-stitching into long overlapping oligonucleotides (Step IV). Many long oligonucleotides and a cloning vector are pooled (Step V) and transformed into yeast (Step VI), which assembles a plasmid that harbors a small ~1 kbp fragment of the mitochondrial genome. In the first cycle, the genome is cloned as small fragments across many plasmids (green; Step VII). The genome is iteratively cloned (Steps V–VII) into progressively fewer fragments by assembling larger fragments from many smaller previously cloned fragments (blue; Step VII). The cycle repeats until a single yeast strain assembles an entire genome (grey; Step VII). Figure created with BioRender.com.

vivo in a host organism, typically *E. coli* or *S. cerevisiae*, to protect and expand the population of cloned DNA.

1.4.2   Host organisms

Many potential organisms can be used as hosts for rDNA, but the most widely used are *E. coli* and *S. cerevisiae* [7]. Innate characteristics of rDNA can often cause unexpected effects on host organisms, such as decreased host cell viability or plasmid stability due to polymerase stalling in repetitive regions of DNA [116,132,133] or aberrant gene expression [114,116,122,134–136] that can drain the host cell's energy stores. This is especially true of mitochondrial genomes that have historically been troublesome to clone and maintain in bacteria [114,115,119,122,124,126,129,135,137]. The likelihood of shared genetic elements between a host genome and rDNA can initially be estimated by the similarity of the G+C%-content of their DNA [1,7,116,122]. For instance, it is more likely that origins of replication, promoters, and terminators will be recognized or occur spontaneously when prokaryotic hosts are used to clone mitochondrial genomes due to their similarity in G+C%-content and prokaryotic ancestry.

Many challenges can be overcome using a eukaryotic host such as *S. cerevisiae*, which has evolved different expression and translation machinery that do not recognize conjugation the prokaryotic genetic elements [7,119,126,138]. However, it may be beneficial to use prokaryote hosts for specific circumstances, such as cell fusion experiments or bacterial conjugation (*in vivo* DNA delivery). Therefore, it is essential to test host burden and plasmid stability before storage in any host organism. Typically, host burden is measured by comparing the growth of strains harboring rDNA to wild-type on plates or in liquid media. Most often, the growth rate will decrease as the plasmid copy number increases [134,139,140]. Whereas, as plasmid size increases, the growth rates are unaffected, but maximum culture cell density decreases [140]. Plasmid stability is tested by isolating plasmids before and after propagation and confirming their integrity by sequencing or enzymatic digestion [114,117,124,126]. If problems are identified, the

causative DNA sequences can be modified or removed to resolve the issue, or a low copy number plasmid vector can be used to reduce metabolic load [114,116,119,122,124].

1.5　Mitochondrial transformation

The introduction of rDNA into a cell is accomplished in three ways: 1) transduction, 2) transformation and 3) bacterial conjugation [112]. Examples of these methods include agrobacterium-mediated DNA transfer, glass bead agitation, biolistic-mediated DNA delivery, electroporation, and cell-penetrating peptides. Many DNA delivery methods have been attempted on model organisms such as, *S. cerevisiae* and *Chlamydomonas reinhardtii*, but only a handful of techniques have successfully introduced rDNA into a mitochondrion [93,141–143]. While electroporation [93,114,144,145] and bacterial conjugation [93,145] have been used to deliver DNA to isolated mammalian mitochondria (Figure 1-3), only biolistic-mediated DNA delivery has transformed intact mitochondria within whole cells [89,141,146–157].

1.5.1　Biolistic-mediated DNA delivery

The biolistic transformation method has been shown to deliver DNA to all cellular compartments, including the nucleus and organelles [6,107,108,150,151,154]. This method accelerates particles coated with DNA toward a cell culture that mechanically passes through the cell membranes, delivering DNA to all cellular compartments (Figure 1-4) [6,149,150,154]. The mechanical shear forces applied to the DNA as it passes the cell membrane and cytoplasm create an inverse relationship between the size of DNA and the success in delivering DNA to the cell. In all eukaryotes (i.e., fungal, mammalian, and plant), this delivery method results in extensive cellular damage killing a vast majority of transformable cells and for mitochondrial transformation is currently limited to cells containing a protective cell wall, such as fungi or algae, that can mitigate much of the cellular damage caused [149,158,159].
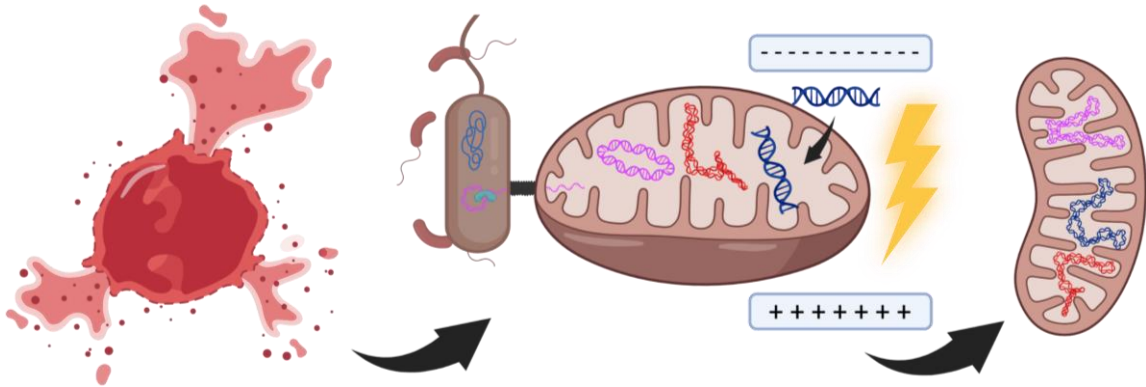
Figure 1-3. Transformation and bacterial conjugation of isolated mitochondria. Functional and structurally intact mitochondria are isolated from cells (left). Extracted mitochondria can be genetically altered using bacterial conjugation or electroporation (middle). The schematic depicts three different DNA molecules from left to right: 1) plasmid DNA (pink), 2) native mitochondrial genome (red), and 3) linear DNA (dark blue). The transformed mitochondria persist transiently and can be studied (right). Figure created with BioRender.com.
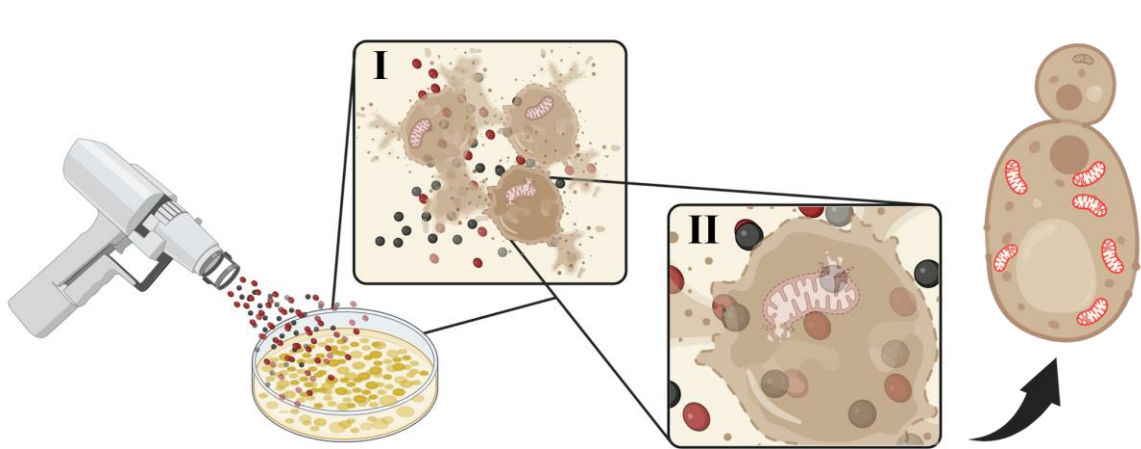
Figure 1-4. Biolistic-mediated DNA delivery. Particles coated with DNA for delivery (red and black) are accelerated by a gene gun (left) towards a petri dish containing densely grown cells. In the first window (I), particles puncture the cell wall and a proportion of organelle compartments, including mitochondria (pink). In the second window (II), a black particle ruptured a mitochondrion and successfully deposited DNA in a now-damaged organelle. A minority of cells successfully repair their cell wall and organelle membranes and re-constitute a cell with transformed mitochondria (red; right). Figure created with BioRender.com.

1.5.2 Bacterial conjugation

Bacterial conjugation is a natural process by which bacteria physically exchange genetic material (bp–Mbp in size). Bacterial donors harbor a conjugative plasmid encoding all the structural and mobilization components of the bacterial conjugation process [160,161]. The structural proteins form a pilus that transverses the donor and recipient cells [160,162–164]. The mobilization proteins recognize and bind the plasmid DNA (pDNA) and actively co-transport it through the pilus to the recipient cell cytoplasm as single-stranded DNA [160,165–171]. Bacterial conjugation has become an established method of *in vivo* DNA delivery to eukaryotes such as yeasts and algae and has previously been shown to deliver DNA to isolated mammalian mitochondria [117,118,145,172–175].

These promising data have encouraged attempts to redirect DNA delivery by bacterial conjugation from the nucleus to the mitochondria as an alternative to the biolistic-mediated DNA delivery method. Bacterial conjugation is an appealing alternative method for development because the DNA is transferred entirely *in vivo*, avoiding DNA damage by shearing that commonly occurs *in vitro* [93,145]. Mitochondria-specific DNA delivery by bacterial conjugation is being explored using 1) cell fusion accompanied by intra-cellular bacterial conjugation [93,112,176,177] and 2) engineering conjugative mobilization proteins with mitochondrial localization signals (Figure 1-5) [93,178,179]. In either scenario, altering bacterial conjugation for efficient mitochondrial DNA delivery will require seizing the host's mitochondrial protein translocation machinery to import foreign bacterial proteins and DNA.

1.6 Emerging and existing platforms

Many eukaryotic platforms are used for synthetic biology, such as plants, fungi, algae, and animal cell lines [1–5,92,180]. Microalgae and yeast are increasingly used eukaryotic systems for synthetic biology because, unlike animals or plants, they are single-celled
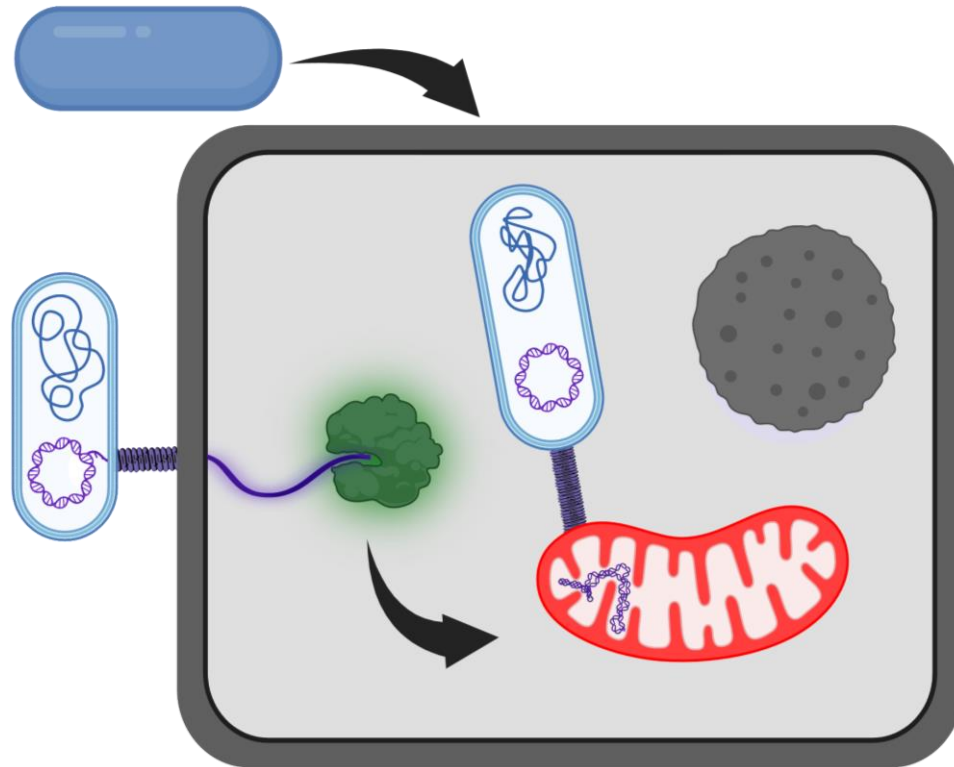
Figure 1-5. Strategies for mitochondrial DNA-delivery by bacterial conjugation.
Two approaches for mitochondria-specific DNA delivery are cell fusion accompanied by
intra-cellular bacterial conjugation (top) and engineering of key conjugative mobilization
proteins with mitochondrial localization signals (bottom). In the first approach, a
bacterium (blue) is fused with a eukaryote and preferentially delivers DNA (purple) to
mitochondria (red) instead of the nucleus (black). In the second approach, a relaxase
engineered with a mitochondrial localization signal (green), transits to the mitochondria,
and delivers DNA to the mitochondrial matrix. Figure created with BioRender.com.

organisms that divide rapidly and can be scaled up in bioreactors [1,92,180,181].
Microalgae are attractive due to their genetic diversity, range of metabolic processes, and
physical characteristics [1,6,92,182]. Yeasts are appealing due to their hardiness in
diverse environments and relevance to human health and industry [180,183–190]. To
fully harness these systems for biotechnology, a synthetic biology approach, including
mitochondrial genome cloning and delivery, is required. Mitochondrial genomes must
first be cloned, which has been completed for several species, including human, mouse,
fungi, plants, and algae [114,115,119,121,123–131]. However, methods for DNA
delivery to the mitochondria of unicellular eukaryotes have been reported for only a few
species: *S. cerevisiae* [146,149–154,157,191], *C. reinhardtii* [147,148,154–156,192,193],
and *Candida glabrata* [194].

In each case of successful mitochondrial DNA transformation, the minimal
requirement was the identification of respiratory deficient mutants that display a
recoverable phenotype [147,149–151,153–155]. These mutants can be identified from
spontaneous mutations or generated by random mutagenesis using ethidium bromide or
acroflavin [89,152,156,195,196]. In the case of *S. cerevisiae* and *C. glabrata*, these were
the petite mutants, which only grow on fermentable media, and in *C. reinhardtii*, the *dum*
mutants that can only grow in the presence of light [154]. Respiratory-deficient mutants
harbor either a large deletion (*rho⁻/dum*), point mutations (*dum*) [89,148,154,156,196], or
lack a mitochondrial genome entirely (*rho⁰*) [149,151,154]. Phenotypically, most *dum*
mutants have lost the ability to grow under heterotrophic conditions; however, some with
altered *nad* genes can grow in the dark but more slowly than wild-type [196,197]. The
result is the availability of a collection of transformable mitochondrial mutants for yeast
and algae.

Petite and *dum* mutants have many properties that make them essential models for
mitochondrial transformation [71,149,154]. First, these mutants survive without
functional mitochondria (*rho⁻/rho⁰/dum*) [149,151,154]. Both the yeast and algae
mitochondria perform active homologous recombination allowing for directed DNA
modifications based on sequence similarities [89,149,150,154]. Further, *rho⁰* mutants can
be transformed with bacterial plasmids and do not require an origin of replication to be

maintained, drastically simplifying engineering efforts [149–151,154]. Finally, the sexual mating of *S. cerevisiae* mitochondrial transformant strains results in homoplasmic cells harboring a mutation of interest [71,149,151,152,154].

1.6.1   *S. cerevisiae*

S. cerevisiae is the most intensively studied model organism for eukaryotic systems, with an extensive genetic toolbox and sequenced genomes [7,102,184,198,199]. Mitochondrial transformation is performed using biolistic-mediated DNA delivery with a transformation efficiency ranging from 0.1–1.3 transformants/µg of DNA [149–151]. The experiment typically co-transforms two DNA molecules, one with nuclear selection and the other with a portion of wild-type mitochondrial DNA [149–154,157]. Following bombardment, the cells are plated on primary selection for the nuclear marker and incubated for 4–5 days [149,151,154,157]. Selection for mitochondrial transformants is performed among the nuclear transformants by mating with a non-respiring tester strain, which results in respiring diploids [149–152,154,157]. Selection markers, such as *URA3* or *TRP1*, when used in the mitochondria, escape to the nucleus at a high frequency [154,200,201], whereas the markers *ARG8*[m] [152,154], *RIP1*[m] [153,154], *FUM*[m] [154], and *GFP*[m] [146,154,157] have been re-coded for expression in the mitochondria and are stably maintained. *S. cerevisiae* is the most developed system for mitochondrial transformation and offers foundational knowledge and methodologies for developing other eukaryotes for mitochondrial genome engineering.

1.6.2   *C. reinhardtii*

The only algal species where mitochondrial transformation has been achieved is *C. reinhardtii*. Mitochondrial transformation is performed using biolistic-mediated DNA delivery with efficiencies ranging from 100–250 transformants/µg of DNA [154,155]. DNA constructs are designed for *dum* mutants that recover their respiratory competency [89,155,156,192]. Following bombardment, colonies are grown in the dark for 4–8

weeks, and then transformants are confirmed by diagnostic PCR [89,147,148,154–156,192,193]. In addition to recovering a heterotrophic phenotype, variants of *C. reinhardtii*'s *cob* gene were identified that confer antibiotic resistance to myxothiazol [202], enabling the transformation of a mutant with intact telomeres [154,155]. Other selection markers, such as *Sh ble* [154,192] and *GFP* [147,154], have since been successfully used. *C. reinhardtii* represents a crucial first step in the mitochondrial transformation of algae and a systematic approach to developing methods in other algae.

1.6.3   Diatoms and unconventional yeasts

Developing interest in algae and yeasts for synthetic biology applications and basic biology research has resulted in efforts to repurpose the existing mitochondrial transformation methods in *S. cerevisiae* and *C. reinhardtii* to additional yeast and algae species. Diatoms are one group of algae that are of particular interest for generating high-value molecules, such as precursors for biofuel, and they are important contributors to the global carbon and silica cycles [1,6,182,203,204]. Despite there being little success of mitochondrial genome engineering in algae species other than *C. reinhardtii* to date, interest is mounting, as demonstrated by the SynDiatom Project, which seeks to create diatomaceous algae composed of synthetic nuclear, chloroplast and mitochondrial genomes [10].

In yeast, the progress made with *S. cerevisiae* presents powerful tools for mitochondrial genome engineering, such as multiple selection markers, transformable knockout strains, maintenance of bacterial plasmids, and mating protocols that generate mitochondrial homoplasmy. Significantly, the occurrence of petite mutants extends to many species beyond *S. cerevisiae*, including *C. glabrata*, *C. albicans*, *Kluyveromyces lactis*, *Saccharomyces castelli*, and others [71,194]. Recently, *C. glabrata*, a closely related species to *S. cerevisiae*, was reported to have undergone successful mitochondrial transformation using the *ARG8*$^{\mathrm{m}}$ marker [194]. Although mitochondrial homoplasmy was not obtained, heteroplasmic transformants were confirmed by PCR, quantitative PCR, and Southern blotting and were stable in the absence of arginine.

Before mitochondrial transformation in diatoms and unconventional yeasts is possible, their mitochondria require proteomic and genetic characterization. Genetic mutations of the mitochondrial genome that produce viable organisms with either antibiotic resistance or recoverable respiratory deficiencies need to be identified and characterized. Standardized selection markers and transformation protocols must be reproduced for multiple algae and yeasts that enable reliable selection of transformants and generation of mitochondrial homoplasmy.

1.7   Scope of this thesis

The foundation of this thesis is to progress the fields of synthetic biology and organelle engineering by improving and standardizing methods for mitochondrial genome engineering and DNA delivery. Synthetic biology seeks total genetic control over biological systems through WGE, which includes the mitochondrial and chloroplast genomes in eukaryotes. However, the development of WGE tools for genome-scale manipulation of DNA is limited to the nucleus. Genetic tools that enable the creation and delivery of designer genomes to any compartment in microbes will empower researchers with potential opportunities, including increasing biosafety and containment of laboratory microbes, producing lucrative biomolecules, and better understanding the origins of eukaryotic life.

The completion of WGE technologies for all eukaryotic genomes will be an unprecedented milestone for synthetic biology and provide a platform to engineer life in astonishing ways yet to be imagined. With WGE tools, the genetic code of microbes can be altered or expanded to form an effective barrier between laboratory and environmental microbes, whereby the genetic code of either species would be misinterpreted by the other. The expression of biosynthetic pathways in mitochondria supports the production of diverse biomolecules by offering an alternative intracellular environment that maintains differing redox potentials or oxygen concentrations. Finally, installing "ancestral-like" mitochondrial genomes will provide insights into how eukaryotic life began and eukaryogenesis evolved into extant eukaryotic species observed today.

Expanding WGE technologies to mitochondrial genomes is vital to harness the potential of eukaryotic platforms for synthetic biology.

Previously, mitochondrial genomes have been cloned in yeast and bacteria with varying degrees of adversity, but few strategies or tools for genome-scale engineering of mitochondrial genomes have been developed and demonstrated. Furthermore, despite progress in cloning and manipulating mitochondrial genomes, methods for delivering DNA to mitochondria are exclusive to three microbes using biolistic-mediated gene gun delivery, which is highly destructive and inefficient. As an alternative, *in vivo* methods of DNA delivery, such as bacterial conjugation, are being adapted for mitochondrial DNA delivery. Previous groups have attempted to improve DNA delivery to eukaryotes using bacterial conjugation by screening the genetic background of donor and recipient cells and optimizing bacterial conjugation parameters. The genetic tools this thesis presents move the field forward by enabling the rapid engineering of entire diatom mitochondrial genomes and improving DNA delivery to eukaryotic cells by bacterial conjugation.

In Chapters 2 and 3, I developed a whole mitochondrial genome engineering platform for two diatoms, *P. tricornutum* and *T. pseudonana*. Each genome was cloned into both *S. cerevisiae* and *E. coli* using PCR-based and TAR cloning methods. I characterized the mutation rate of each cloning method and performed a cycle of engineering on a mitochondrial genome to demonstrate the speed and ease of introducing genetic modifications. I assessed the host burden of *S. cerevisiae* and *E. coli* harboring these genomes via growth rates and plasmid stability in *E. coli*. Finally, gene expression from the mitochondrial plasmids in *E. coli* was analyzed using RNA sequencing.

Then I focused my attention on improving DNA delivery methods to eukaryotic cells via bacterial conjugation in Chapter 4. I aimed to enhance bacterial conjugation rates to establish a practicable *in vivo* DNA delivery method more amenable to mobilization protein engineering efforts. Using the pTA-Mob 2.0 conjugative plasmid, I developed a deletion plasmid library containing 55 deletions and tested each plasmid for DNA delivery to bacterial and eukaryotic cells. Deletions were combined into cluster deletions to generate progressively smaller conjugative plasmids, and a superior

conjugative plasmid was created: pSuperCon5 (pSC5). I showed that the improved conjugative ability of pSC5 was donor independent by using *Sinorhizobium meliloti* as a donor rather than *E. coli*. My colleagues and I repurposed bacterial conjugation by "loading" pSC5 with toxic genes for killing yeasts as a proof-of-principle for a novel antifungal. More importantly, we demonstrated that bacterial conjugation with pSC5 could deliver DNA to unconventional yeasts, including *C. auris* and *Metschnikowia* species, which had not previously been reported. Together these resources for mitochondrial genome engineering and improved DNA delivery should improve the feasibility of future endeavors in whole mitochondrial genome engineering.

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Chapter 2

2 Rapid method for generating designer algal mitochondrial genomes

The work presented in this chapter is adapted from:

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2.1 Introduction

Pressing challenges in agriculture, medicine, and energy can be addressed using designer organisms with engineered traits. The promise of synthetic biology lies in the ability to build, deliver, install, and test synthetic genomes and biosynthetic pathways in specialized hosts. *Phaeodactylum tricornutum* is a model diatom algal species that is an attractive candidate for synthetic biology applications [1–5]. For example, *P. tricornutum* is a popular candidate for biofuel production due to its natural propensity for lipid storage [6]. Due to the industrial and academic interest in this algal species, nuclear, mitochondrial, and plastid genomes were sequenced [7–9]. The availability of genome sequences has allowed for the development of genetic tools and DNA delivery methods such as biolistic-mediated transformation [10,11], electroporation [12–14], and bacterial conjugation [15,16]. Additional tools for *P. tricornutum* include a method for cloning whole chromosomes in yeast and *Escherichia coli* [17], characterized centromeres for maintaining episomal DNA [18], and genome-editing technologies [19–22]. We now have a powerful arsenal of tools for engineering algal nuclear genomes; however, tools for engineering and delivering organelle genomes are still lacking.

There are several advantages to engineering organelle genomes and installing synthetic DNA in these compartments rather than the nucleus. First, the polycistronic gene organization, lack of transgene silencing, and reduced positional effects in

organellar DNA simplify genome engineering relative to the nucleus. Second, organelles allow for the compartmentalization of biosynthetic pathways, which confines toxic intermediates, increases the metabolic flux, and minimizes competing reactions from the rest of the cell [23]. To exploit these benefits, scientists have cloned whole organelle genomes from various organisms, including human, mouse, maize, rice, and some algae [24–29]. Yet, a rapid method for whole organelle genome engineering for eukaryotic algae has not been established.

The first obstacle is the need for a fast and inexpensive method for synthesizing, assembling, and storing synthetic organelle genomes. While processes for synthesizing and replacing nuclear genomes have been established for some prokaryotes [30–34] and eukaryotes [35], genome-scale engineering and delivery are still very challenging [36]. The large, repetitive elements and AT-rich sequences of organelle genomes complicate the synthesis and cloning processes. Spurious expression of cloned genomes can be toxic to host organisms, including *Saccharomyces cerevisiae* and *E. coli* [37]. Importantly, the targeted delivery of whole organelle genomes to the appropriate cellular compartment in eukaryotic algal cells is still not possible.

Here, we report a rapid protocol for cloning designer *P. tricornutum* mitochondrial genomes with sizes ranging from ~60–95 kbp and demonstrate their maintenance in eukaryotic and prokaryotic host strains as the first step of a platform for robust, genome-scale mitochondrial genome engineering. We used TAR cloning to capture the wild-type mitochondrial genome of *P. tricornutum* and developed a PCR-based approach to clone modified versions of the genome. We then demonstrated the maintenance of wild-type and modified mitochondrial genomes in *S. cerevisiae* and *E. coli*.

2.2   Results and Discussion

2.2.1   Design-build-test cycle to enable rapid engineering of organelle genomes

Currently, there are no established methods for replacing whole eukaryotic algal organelle genomes. To address this need and enable full use of the organelle compartment for installing synthetic genomes, we are developing a design-build-test cycle for efficient capture, manipulation, delivery, and installation of organelle genomes (Figure 2-1A).

In one iteration of the design and build stages of the cycle, we cloned a whole mitochondrial genome and a reduced version with a repetitive region removed. To capture the whole mitochondrial genome, TAR cloning was used (Figure 2-1B). For TAR cloning, total algal DNA was captured in agarose plugs to obtain isolated, intact organelle genomes. Next, the mitochondrial DNA was linearized by a restriction enzyme that recognizes a single cut-site in the targeted mitochondrial genome. If there are no unique restriction enzyme cut-sites available, it is possible to select a restriction enzyme that cuts in multiple locations and perform a partial digest to obtain a proportion of genomes with a single cut at the desired location. If the cut site resides within an essential gene, a modified version (lacking the restriction enzyme cut site) of the gene can be added to the plasmid backbone. Alternatively, once the genome is cloned in yeast, the plasmid backbone can be moved to a different location by co-transforming a plasmid containing homology hooks to the new location and another fragment that will delete the plasmid in the original location and restore the interrupted gene. Finally, a clustered regularly interspaced palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system can be devised to produce appropriate cut-sites at the desired location [39,40].

The linearized algal mitochondrial genome was captured by transforming it into *S. cerevisiae* along with a PCR-amplified plasmid backbone containing homology on each end to the mitochondrial genome regions flanking the cut-site. Although the process is more time-consuming, an entire organellar genome can be cloned. In PCR-based cloning
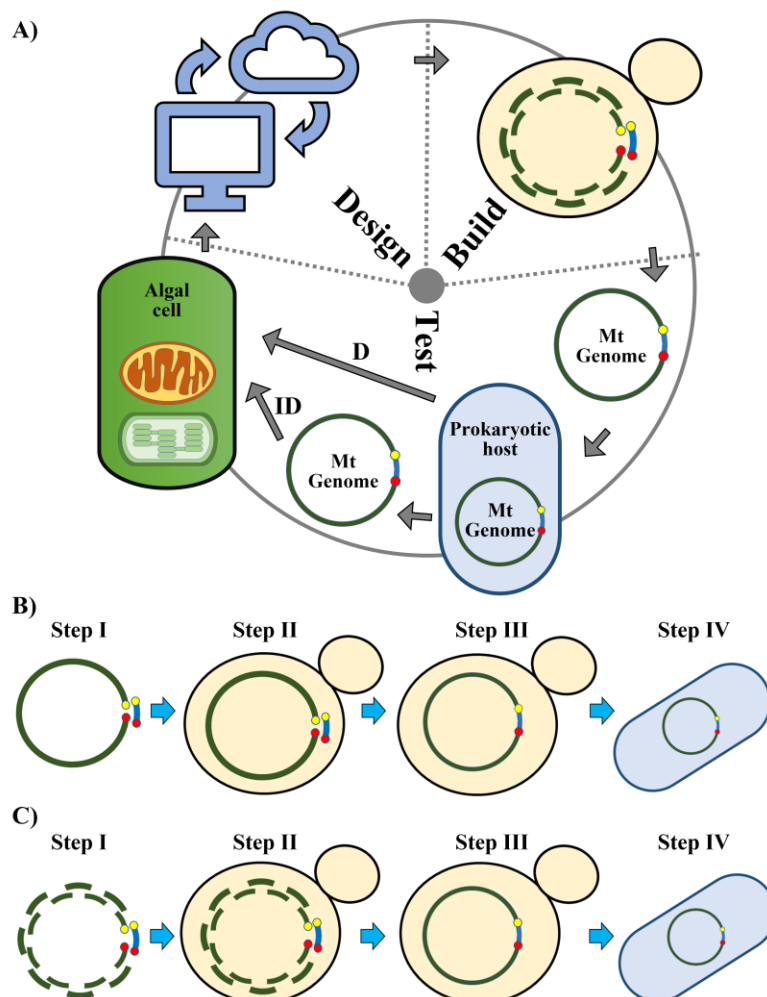
Figure 2-1: Design-build-test cycle for the rapid engineering of mitochondrial genomes. A) Genomes will be designed based on existing knowledge and discoveries from previous cycles; in the build stage, genomes will be synthesized, then assembled and cloned in yeast; in the test stage, genomes will be isolated from yeast, moved to an intermediate prokaryotic host and delivered directly (D) (e.g., bacterial conjugation, cell fusion) or indirectly (ID) (e.g., electroporation, biolistic-mediated transformation) to the mitochondria to test for viability, function, and localization. Mt – mitochondrial. **B–C)** Schematic of the approaches used to clone the mitochondrial genomes of *P. tricornutum*. **B)** Transformation-associated recombination (TAR) cloning method; **C)** PCR-based cloning method. Step I differs between the PCR-based and TAR cloning methods where, for the PCR-based cloning method, multiple overlapping fragments (green) are amplified, while for the TAR cloning method, the mitochondrial genome is linearized at a specific location. For both methods, the plasmid backbone (blue) contains homology overlaps (yellow and red) to the appropriate fragments or location in the genome. Steps II–IV are the same for both methods including DNA transformation (Step II), assembly in *S. cerevisiae* via homologous recombination (Step III), and transfer of cloned genomes into *E. coli* (Step IV).

(Figure 2-1C), the mitochondrial genome was cloned indirectly by amplifying fragments from total algal DNA with homologous DNA overhangs to adjacent fragments, followed by transformation into *S. cerevisiae* with the plasmid backbone. This process has allowed for the fast assembly of large plasmids [41]. However, it also has some drawbacks, including the risk of introducing unwanted point mutations and difficulty amplifying larger repetitive elements, which could limit the practicality and versatility of this approach. In both methods, elements required for replication in yeast (e.g., *CEN6-ARSH4-HIS3*) and *E. coli* (e.g., the origin of replication and selectable marker) must be provided as one or multiple plasmid backbone fragments containing appropriate homologous overhangs.

2.2.2   Cloning of the *P. tricornutum* mitochondrial genome

Using the PCR-based approach, we cloned a reduced version of the *P. tricornutum* mitochondrial genome (Figure 2-2A). The wild-type genome was PCR-amplified in eight overlapping fragments that excluded a 35 kbp region of direct repeats [8]. In place of this repeat region, two fragments with genetic elements required for replication and selection in *S. cerevisiae*, *E. coli*, and algae (nuclear localization) were amplified from the multi-host shuttle plasmid pAGE3.0 [16]. In total, 10 DNA fragments were amplified (Figure 2-2B) and assembled following transformation and homologous recombination in *S. cerevisiae* spheroplasts, yielding 5023 transformed yeast colonies (Table 2-1). After moving the assembled plasmids to *E. coli*, two clones, pPT-PCR C1 and C2, were validated by multiplex (MPX) PCR and restriction digests (Figure 2-2C, D) were sequenced and analyzed for mutations (Section 2.2.3).

Importantly, we wanted to assess the fidelity of the PCR-based cloning method by comparing cloned sequences to their parental reference. However, the amplified fragments initially used in this iteration were derived from a potentially heterogenic population of mitochondrial genomes, which may differ from the published sequence. Therefore, to directly determine the mutation rate of the cloning method, the
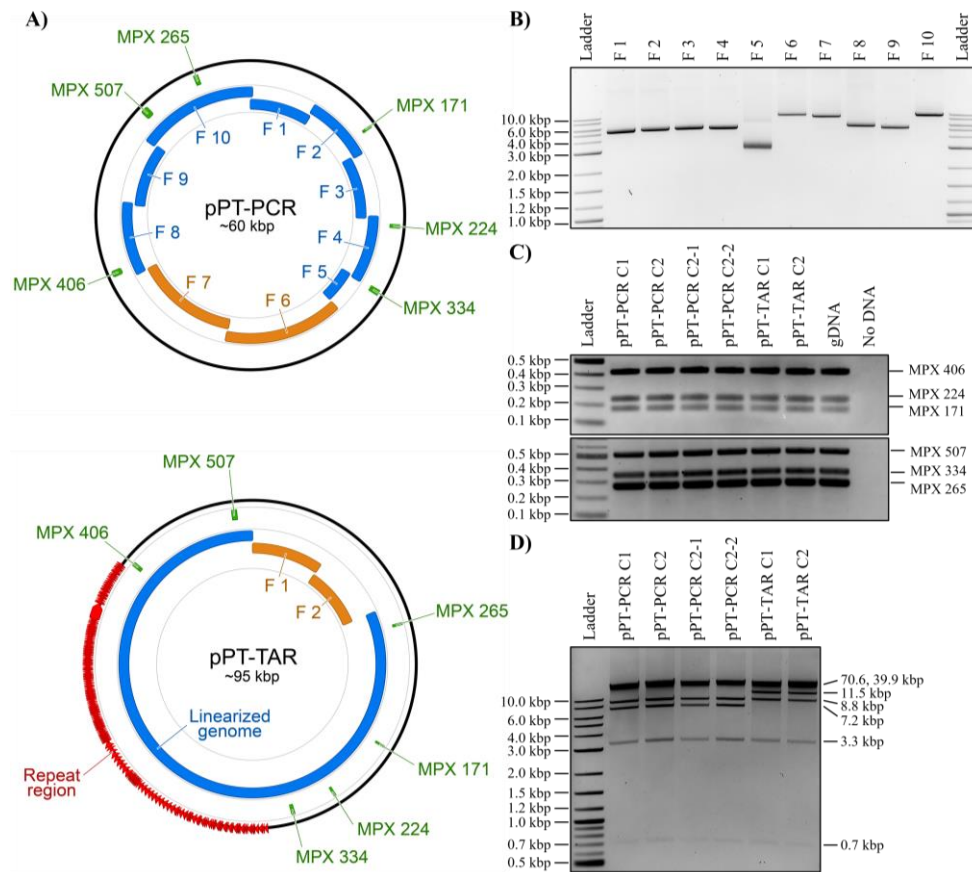
Figure 2-2. Design, amplification, and analyses of cloned *P. tricornutum* mitochondrial genomes. A) Plasmid maps of the *P. tricornutum* mitochondrial genomes cloned using PCR-based cloning (top) or transformation-associated recombination (TAR) cloning (bottom). For the PCR-based cloning method, the relative sizes and positions of the eight mitochondrial fragments (blue) and the two plasmid backbone fragments (orange) are shown. For the TAR cloning method, the relative sizes and positions of the linearized genome (blue) and the two plasmid backbone fragments (orange) are shown. In addition, the six MPX PCR amplicons used for diagnostic screening are indicated (green). These images were generated using Geneious version 2020.0, created by Biomatters. **B)** Agarose gel electrophoresis of the 8 PCR-amplified fragments of the *P. tricornutum* mitochondrial genome (fragments 1–5 and 8–10) and the pAGE3.0 backbone (fragments 6 and 7) for PCR-based cloning. The resulting amplicon sizes for fragments 1–10 are 5217-, 5190-, 5197-, 5175-, 2765-, 9124-, 8571-, 5605-, 5299-, and 9264-bp, respectively. **C)** MPX PCR screen of six cloned algal mitochondrial genomes isolated from *E. coli*. The expected size for each MPX amplicon is indicated by its name (in bp). **D)** Diagnostic restriction enzyme digest of six cloned algal mitochondrial genomes using SrfI and SacII. For the pPT-PCR clones, the expected band sizes are 39,925-, 8758-, 7177-, 3276-, and 707-bp; and for the pPT-TAR clones, the expected band sizes are 70,647-, 11,506-, 8758-, 3276-, and 707-bp. Notes: 1) There was no observable size difference between the 70.6 kbp and 39.9 kbp fragments, which is most likely due to the electrophoresis conditions (1% agarose gel, 100 V for 90 min); 2) The 707-bp band is present but very faint. For all gels, we used NEB 2-log ladder.

Table 2-1: Cloning of the *P. tricornutum* mitochondrial genome in the host organisms *S. cerevisiae* and *E. coli*. Two PCR-based cloning assemblies and one transformation-associated recombination (TAR) cloning assembly were performed. For the *E. coli* selection media, CM indicates chloramphenicol antibiotic. For the *S. cerevisiae* selection media, -HIS indicates synthetic complete media lacking histidine.

Design Assembly type DNA source	*S. cerevisiae*			*E. coli*			
	Selection media	Colony count	MPX PCR screen	Selection media	Selected yeast colony: *E. coli* colony count	MPX PCR screen	Final genomes selected for analysis
Reduced genome PCR – 10 fragments Total DNA	-HIS	5023	20/20	CM	Yeast colony C1: 30 Yeast colony C2: 18	C1 = 8/8 C2 = 8/8	pPT-PCR C1 pPT-PCR C2
Reduced genome PCR – 10 fragments Clone pPT-PCR C2	-HIS	4880	20/20	CM	N/D	C2-1 = 5/5 C2-2 = 5/5	pPT-PCR C2-1 pPT-PCR C2-2
Full genome TAR – 3 fragments Digested total DNA	-HIS	608	2/204	CM	Yeast colony C1: 119 Yeast colony C2: 46	C1 = 1/1 C2 = 2/2	pPT-TAR C1 pPT-TAR C2

mitochondrial genome was re-amplified using DNA isolated from an isogenic culture of *E. coli* carrying pPT-PCR C2. The 10 fragments were assembled using the same method, with similar results as the first assembly (Table 2-1, Section 2.2.3). Two additional clones, pPT-PCR C2-1, and C2-2, were selected for further analysis.

We used the TAR cloning-based approach to clone the complete, repeat-containing genome (Figure 2-2A). Total *P. tricornutum* DNA was digested with the restriction enzyme PvuI, which cuts the mitochondrial genome at a single site. We amplified the plasmid backbone from pAGE3.0 as two fragments with end homology to each other and the sequences flanking the mitochondrial PvuI cut site. Transforming both PCR-amplified plasmid backbone fragments and the linearized genome into *S. cerevisiae* spheroplasts yielded 604 colonies (Table 2-1), from which 204 were screened and two clones, pPT-TAR C1, and C2, were identified as positive for the presence of the mitochondrial genome using MPX PCR. We then transferred these genomes to *E. coli* and reanalyzed them with MPX PCR and diagnostic restriction digest (Figure 2-2C, D). Although the build stage using the TAR cloning approach was successful, it was time-consuming and labor-intensive due to the requirement of obtaining high-quality DNA and screening a larger number of colonies. Nonetheless, this iteration can act as a good template for future design-build cycles, particularly for cloning large organellar genomes that are difficult to amplify by PCR.

2.2.3   Sequence analysis of cloned *P. tricornutum* mitochondrial genomes

The six selected *P. tricornutum* mitochondrial clones were sequenced and analyzed for mutations. Sequences obtained for the original PCR-cloned (pPT-PCR C1 and pPT-PCR C2) and TAR cloned (pPT-TAR C1 and pPT-TAR C2) plasmids were aligned to reference sequences based on the published sequences [8,16]. The reassembled PCR-cloned plasmids (pPT-PCR C2-1 and pPT-PCR C2-2) were aligned to the sequence of their parent clone, pPT-PCR C2. Upon analyzing pPT-PCR C1 and C2, an average of 7 mutations per 60 kbp was found (Table 2-2), corresponding to 1 mutation per 8.6 kbp.

Table 2-2: Summary of mutations identified in the cloned *P. tricornutum* mitochondrial plasmids. Identified mutations are categorized as point mutations (synonymous, missense, nonsense, and those found in non-coding regions) or gap mutations (insertions and deletions, either non-coding or coding).

Clone	Point mutations				Gap mutations				Total
	Synonymous	Missense	Nonsense	Non-coding	Non-coding		Coding		
					Insertions	Deletions	Insertions	Deletions	
pPT-PCR C1	0	4	0	2	1	1	1	0	9
pPT-PCR C2	0	3	1	0	0	1	0	0	5
pPT-PCR C2-1	0	3	0	2	0	0	0	0	5
pPT-PCR C2-2	0	1	0	0	0	0	0	0	1
pPT-TAR C1	1	0	0	0	0	0	0	0	1
pPT-TAR C2	0	0	0	7	1	8	0	0	16

The two reassembled clones, pPT-PCR C2-1 and C2-2, acquired an average of 3 mutations per 60 kbp (Table 2-2), corresponding to 1 mutation per 20 kbp. See Supplementary Table B-2 for additional information about specific mutations. pPT-TAR C1 had only a single synonymous substitution (Table 2-2). However, the other clone generated using this method, pPT-TAR C2, contained a series of deletions in the repetitive region of the *P. tricornutum* mitochondrial genome (Table 2-2). This clone also carried eight single nucleotide substitutions, albeit all in non-coding regions of the plasmid except a synonymous mutation in the *cox3* gene. The difference in mutation rate between these two plasmids could be the result of recombination of the highly repetitive region of the mitochondrial genome during TAR cloning or an artifact of the sequencing method used as resolving large repetitive regions may require the use of long-read sequencing technologies.

Genetic changes found using both cloning methods could have occurred during the cloning process of each mitochondrial plasmid or during propagation in the host organisms harboring the plasmids. It is also plausible that some of these variants could naturally exist in the population of *P. tricornutum* mitochondrial genomes. If desired, identified mutations could be fixed through additional iterations of the design-build-test cycle. If necessary individual fragments would be cloned, and the sequence confirmed before being used in yeast assembly.

2.2.4   Maintenance of *P. tricornutum* mitochondrial plasmids in host organisms

S. cerevisiae and *E. coli* were used as host organisms to capture and store the *P. tricornutum* mitochondrial genome. *S. cerevisiae* was chosen because it is currently the best organism for assembling large DNA molecules due to its highly efficient homologous recombination machinery and demonstrated ability to maintain a wide range of genomes without adverse effects [42]. However, when using standard protocols for isolating pDNA, the yields from *S. cerevisiae* tend to be low. To overcome this problem, after assembly in *S. cerevisiae*, the cloned mitochondrial plasmids were transformed into

E. coli. In *E. coli*, the mitochondrial plasmids, which contain an arabinose-inducible origin of replication, can be induced to a higher copy number to generate increased DNA yields. Importantly, propagating genomes in *E. coli* allows for the development of direct transfer methods, such as bacterial conjugation, to deliver the plasmids to the desired destination organism. At the same time, because mitochondrial genomes are hypothesized to be of prokaryotic origin, there is a chance that a prokaryotic host would express functional proteins that might have adverse or toxic effects [32,43].

We sought to examine the burden of propagating the cloned mitochondrial genomes in eukaryotic and prokaryotic host strains. Colony formation on selective plates and growth rates in liquid media for both *S. cerevisiae* and *E. coli* strains carrying these plasmids were evaluated. If a plasmid causes cellular stress, it can decrease the growth rate and form smaller or fewer colonies on the plate. In *S. cerevisiae*, there was no observable decrease in colony size when strains harboring cloned mitochondrial genomes were dilution spot plated, compared with control plasmids with the same backbone, pAGE3.0 and pPtGE31 [19] (Figure 2-3A). Next, we compared growth in liquid media by using a 96-well plate reader. Interestingly, the strains carrying the mitochondrial genomes grew faster in liquid compared to the pAGE3.0 control but at a similar rate to the pPtGE31 control (Figure 2-3C and Table 2-3). Overall, this indicates that the maintenance of *P. tricornutum* mitochondrial genome plasmids do not have an adverse effect on yeast growth. In *E. coli*, we did not observe any substantial negative effects on growth rates of cells harboring *P. tricorunutm* mitochondrial genomes compared to the control plasmids (Figure 2-3B, D, and Table 2-3). In addition, there was no substantial effect on growth rates when the cells were induced to have a high copy number of mitochondrial genomes. In future studies, additional experiments should be performed to evaluate the expression of all mitochondrial genes in *S. cerevisiae* and *E. coli*.
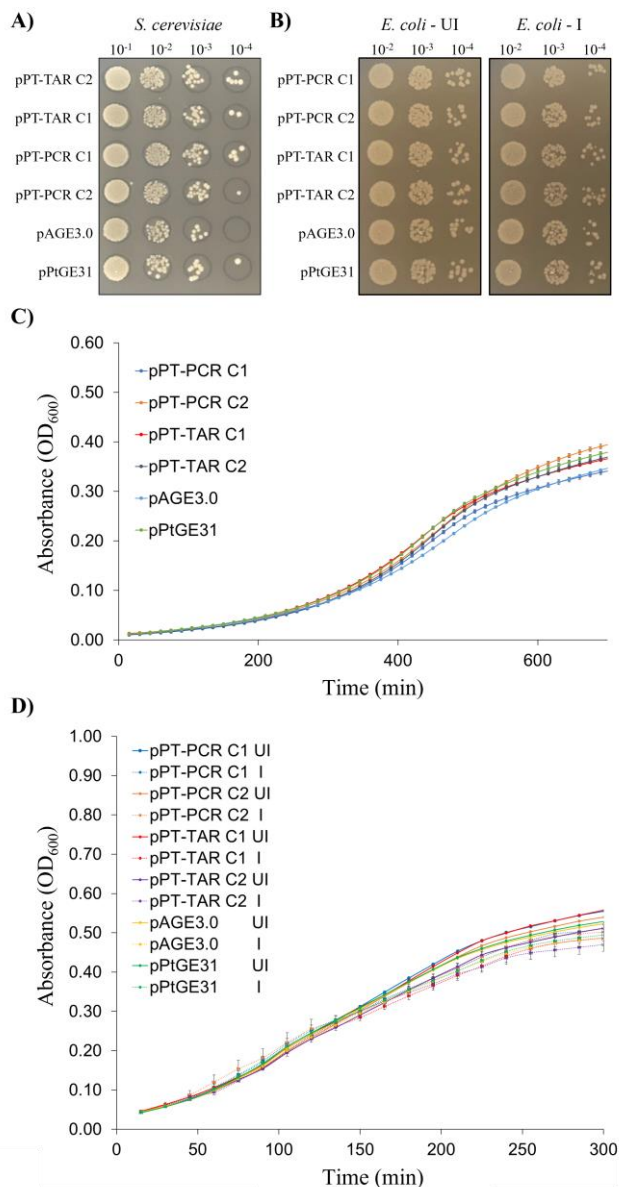
Figure 2-3. Analysis of growth of *S. cerevisiae* and *E. coli* strains harboring cloned *P. tricornutum* mitochondrial genomes on solid media and in liquid media. A) Dilutions (10^{-1}–10^{-4}) of *S. cerevisiae* strains plated on solid synthetic complete media lacking histidine supplemented with adenine (60 mg L^{-1}). **B)** Dilutions (10^{-2}–10^{-4}) of *E. coli* strains plated on solid LB media supplemented with chloramphenicol only (UI - uninduced) or with chloramphenicol and arabinose (I - induced) to increase plasmid copy number. **C)** Growth curves of *S. cerevisiae* strains grown in liquid synthetic complete media lacking histidine supplemented with adenine (60 mg L^{-1}). For each strain (n = 4) and error bars represent the standard error of the mean. **D)** Growth curves of *E. coli* grown in liquid LB media supplemented with chloramphenicol only (UI) or with chloramphenicol and arabinose (I). For each strain (n = 8) and error bars represent the standard error of the mean. td: doubling time.

Table 2-3. Doubling time of host organisms harboring either a mitochondrial genome or control plasmid. Doubling times of plasmid-containing *S. cerevisiae* strains were grown in synthetic complete yeast media lacking histidine at 30 °C with continuous, double orbital shaking. Doubling times of plasmid-containing *E. coli* strains were grown in either LB media supplemented with chloramphenicol (15 μg mL^{-1}; Uninduced) or LB media supplemented with chloramphenicol (15 μg mL^{-1}) and arabinose (100 μg mL^{-1}; Induced) at 37 °C with continuous, double orbital shaking.

Sample	Doubling Time (min)		
	S. cerevisiae	*E. coli*	
		Uninduced	Induced
pPT-PCR C1	71	37	40
pPT-PCR C2	76	38	40
pPT-TAR C1	72	39	41
pPT-TAR C2	71	39	39
pAGE3.0	82	37	37
pPtGE31	72	37	38

2.3   Conclusions

The biotechnological potential of organelle engineering is held back by the need for more reliable methods to replace organellar genomes. Towards the goal of establishing a design-build-test cycle for genome-scale organelle engineering, we have developed two adaptable methods for cloning and manipulating eukaryotic mitochondrial genomes ranging in sizes from ~60–95 kbp. With a PCR-based cloning approach, we cloned a variant of the *P. tricornutum* mitochondrial genome lacking its 35 kbp repeat region, and with a TAR-based cloning approach, we captured the complete genome. The former had a mutation rate ranging from 1 mutation per 8.6–20 kbp, while the latter allowed us to identify one clone with only a single mutation. The cloned genomes imposed no substantial growth burden on *S. cerevisiae* and *E. coli* when these host organisms were used to propagate the plasmids. In this study, we completed the first step in developing a reproducible set of methods for cloning, manipulating, and installing synthetic organelle genomes.

2.4   Materials and Methods

2.4.1   Strains and growth conditions

Phaeodactylum tricornutum (Culture Collection of Algae and Protozoa CCAP 1055/1) was grown in synthetic seawater (L1 media) without silica at 18 °C under cool white fluorescent lights (75 µE m^{-2} s^{-1}) and a photoperiod of 16 h light: 8 h dark. L1 media was made as previously described [15]. *Saccharomyces cerevisiae* VL6−48 (ATCC MYA-3666: *MATα*, *his3-Δ200*, *trp1-Δ1*, *ura3−52*, *lys2*, *ade2−101*, *met14*, psi^{+}, cir^{0}) was grown at 30 °C in rich yeast media (2 x YPDA: 20 g L^{-1} yeast extract, 40 g L^{-1} peptone, 40 g L^{-1} glucose, and 200 mg L^{-1} adenine hemisulfate), or synthetic complete media lacking histidine supplemented with adenine (60 mg L^{-1}). Solid yeast media contained 2% agar. After spheroplast transformation, all complete minimal media used contained 1 M sorbitol [38]. *Escherichia coli* (Epi300, Lucigen) was grown at 37 °C in Lysogeny Broth (LB) supplemented with chloramphenicol (15 µg mL^{-1}).

2.4.2   DNA preparation

2.4.2.1   Isolations of *P. tricornutum* DNA in agarose plugs

For TAR cloning, total DNA was isolated from *P. tricornutum* in agarose plugs. *P. tricornutum* (1.0×10^7 cells mL^{-1}) was plated and grown on 1% agarose L1 plates for four days. Next, 1 mL of 1 M sorbitol was used to scrape algal cells from each plate. Next, the combined cell suspension was centrifuged at 2000 x RCF for 2 min in a 15 mL conical Falcon tube, followed by 3000 x RCF for 1 min. Then, the supernatant was removed, and the cell pellet was resuspended in 2 mL of SPEM solution (1 M sorbitol, 10 mM EDTA (pH 7.5), $Na_2HPO_4 \cdot 7 H_2O$ (2.08 g L^{-1}), $NaH_2PO_4 \cdot 1 H_2O$ (0.32 g L^{-1})) and incubated for 1 min at 37 °C. Next, the resuspended cells were incubated for 5 min at 50 °C and then mixed with an equal volume of 2.2% low-melting point agarose in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA), which was also kept at 50 °C. Aliquots of 100 μL were transferred into plug molds and allowed to solidify for 10 min at 4 °C. Once solidified, the plugs were removed from the molds and transferred into 50 mL conical Falcon tubes containing 5 mL of protoplasting solution ((8.5 mL of SPEM solution, 500 μL zymolyase-20 T solution (50 mg mL^{-1}), 500 μL lysozyme (25 mg mL^{-1}), 500 μL hemicellulase (25 mg mL^{-1}), 50 μL β-mercaptoethanol (14.3 M)) and incubated for 30 min at 37 °C to digest algal cell walls. After, the protoplasting solution was removed, and the plugs were incubated with 5 mL of Proteinase K solution (100 mM EDTA (pH 8.0), 0.2% sodium deoxycholate, 1% sodium lauroyl sarcosine, and 1 mg mL^{-1} Proteinase K) for 24 h at 50 °C. Then, the plugs were washed four times as follows: twice with 25 mL of wash buffer (20 mM Tris and 50 mM EDTA (pH 8.0)) for 2 h each at room temperature (RT), once with 25 mL of wash buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) for 2 h at RT, and a final wash with 25 mL of wash buffer for 2 h.

For PvuI restriction digestion, the plugs were placed in 1.5 mL microcentrifuge tubes and washed with 1 mL of 0.1 x wash buffer for 1 h at RT, followed by a 5 h wash in 1.0 x NEBuffer 3.1 restriction buffer at RT. Finally, each plug was incubated with 50 units mL^{-1} of PvuI restriction enzyme in 1 mL of 1.0 x NEBuffer 3.1 restriction buffer for 4 h at 37 °C. Following the digest, the plugs were washed for 1 h in 1 mL of TE buffer

(pH 8). Next, the TE buffer was removed, a fresh 100 μL of TE buffer (pH 8) was added, and plugs were melted for 10 min at 65 °C. The solution was then equilibrated to 42 °C for 10 min before adding 2 μL (2 units) of β-agarase. Finally, the solution was incubated at 42 °C for 1 h to allow the agarose to be digested. On average, each plug yielded a DNA concentration of 300 ng μL^{-1}.

2.4.2.2   DNA isolation by modified alkaline lysis

Total DNA from *E. coli*, *S. cerevisiae*, and algae were isolated as previously described [15]. Before isolating DNA from *E. coli* for diagnostic restriction digests or sequencing, cells were induced with arabinose. For plasmid induction, 5 mL of *E. coli* overnight cultures grown in LB media supplemented with chloramphenicol (15 μg mL^{-1}) were diluted 1:50 into 50 mL of LB media supplemented with chloramphenicol (15 μg mL^{-1}) and arabinose (100 μg mL^{-1}) and grown for 8 h at 37 °C.

2.4.3   DNA fragment preparation for PCR-based cloning

PCR amplification of mitochondrial fragments was performed using *P. tricornutum* total DNA (first iteration) or *P. tricornutum* mitochondrial DNA cloned on a plasmid and isolated from *E. coli* as described in Section 2.4.2.2 (second iteration) as template DNA. The mitochondrial genome was amplified as eight overlapping fragments (primers: BK 141–144F/R, 145F/250R, 247F/146R, 147F/R, and 148F/140R, listed in Supplementary Table B-1), as well as two additional fragments (primers: BK 88F/245R and 251F/88R, listed in Supplementary Table B-1) to amplify the pAGE3.0 plasmid [16]. The pAGE3.0 plasmid contains all the genetic elements required for selection and stable propagation in host organisms *S. cerevisiae*, *P. tricornutum* (nuclear localization), *E. coli*, and *Sinorhizobium meliloti*. In addition, this plasmid contains an origin of transfer (*oriT*) to allow for plasmid transfer using bacterial conjugation. All primers were manually designed. Forward and reverse primers for fragments 1–4, 9–10, the forward primer for fragment 5, and the reverse primer for fragment 8 were designed to be 40 bp long.

Primers 80 bp in length were designed for fragments 6 and 7, the reverse primer for fragment 5, and forward primer for fragment 8. Overlaps between fragments were between 68 and 200 bp to allow for efficient yeast assembly.

Each fragment was individually amplified in a 25 μL PCR reaction using 1 μL of PrimeSTAR GXL polymerase, 1 μL of template DNA (10–100 ng μL^{-1} isolated total DNA from either *P. tricornutum* or *E. coli*), and the respective forward and reverse primers each at a final concentration of 0.2 μM. The thermocycler was programmed as follows: five cycles of 98 °C for 10 s, 50 °C for 15 s, and 68 °C for 480 s, followed by 25 cycles of 98 °C for 10 s, 55 °C for 15 s, and 68 °C for 480 s, and one cycle of 68 °C for 600 s, finishing with an infinite hold at 12 °C. PCR product amplification was confirmed by performing agarose gel electrophoresis with 2 μL of PCR product on a 1.4% agarose (*w/v*) gel.

To eliminate plasmid template DNA, PCR products were treated with 10 units (0.5 μL) of DpnI restriction endonuclease, incubated at 37 °C for 30 min, and deactivated for 20 min at 80 °C. Fragments were then purified using the EZ-10 Spin Column PCR Products Purification Kit and were combined into a single 1.5 mL microcentrifuge tube to equimolar concentrations (~200 ng of each fragment) and a total volume of ~30 μL.

2.4.4   DNA fragment preparation for transformation-associated recombination (TAR) cloning

P. tricornutum's total DNA was digested with PvuI restriction enzyme, as described in Section 2.4.2.1. The pAGE3.0 plasmid was amplified as two fragments (primers: BK 88F/93R & 92F/88R, listed in Supplementary Table B-1). Each fragment was individually amplified in a 20 μL PCR reaction using 0.8 μL PrimeSTAR GXL polymerase, 0.8 μL of template DNA (10 ng μL^{-1} of plasmid template DNA isolated from *E. coli*), and the respective forward and reverse primers at a final concentration of 0.2 μM. The thermocycler conditions used were as follows: 30 cycles of 98 °C for 10 s, 62 °C for 15 s, and 68 °C for 150 s, one cycle of 68 °C for 600 s, and an infinite hold at 12

°C. PCR product amplification was confirmed by performing agarose gel electrophoresis with 1 μL of PCR product on a 1.4% agarose (*w/v*) gel.

To eliminate the template DNA from the PCR products, DpnI treatment, and purification were performed as described in Section 2.4.3. Then, the fragments were combined into a single 1.5 mL microcentrifge tube in the following proportions: 10 μL linearized genome (~300 ng μL^{-1}) and 7 μL for each plasmid backbone fragment (~300–500 ng μL^{-1}) prior to yeast transformation.

2.4.5   Yeast spheroplast transformation protocol

Yeast spheroplasts were prepared as previously described [38], with the modification that mixtures of DNA fragments were used rather than bacterial culture. After transformation and recovery, the 1 mL of yeast cells was split into 300 μL and 700 μL. Each aliquot was added to a 15 mL conical Falcon tube containing 8 mL of melted 2% agar yeast synthetic complete media lacking histidine supplemented with adenine (60 mg L^{-1}) and 1 M sorbitol, which had been equilibrated at 50 °C. After 4–6 gentle inversions, the mixture was poured on top of an agar plate containing 10 mL of 2% agar yeast synthetic complete media lacking histidine supplemented with adenine (60 mg L^{-1}) and 1 M sorbitol. The plate was then incubated at 30 °C for 3–5 days until yeast colonies emerged for screening.

2.4.6   *E. coli* transformation

TransforMax Epi300 electrocompetent *E. coli* cells were thawed on ice for 20 min. Then, 20 μL aliquots of *E. coli* cells were transferred to sterile 1.5 mL microcentrifge tubes and mixed with 1 μL of total DNA extracted from a single yeast colony [37]. A Gene Pulser Xcell Electroporation System was set to 25 μF capacitance, 200 Ω resistance, and 2.5 kV voltage. The DNA mixture and *E. coli* was then transferred to an ice-cold 2 mm cuvette and electroporated. Immediately, 1 mL of super optimal broth with catabolite

repression (SOC; 20 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 0.5 g L^{-1} NaCl, 10 mL of 250 mM KCl, 5 mL of 2 M MgCl$_2$, and 20 mL of 1 M glucose) media was added to the cuvette, which was then incubated at 37 °C for 30 min, without shaking. Next, the mixture was transferred to a sterile 1.5 mL microcentrifuge tube and incubated for 1 h at 37 °C, shaking at 225 RPM. Finally, 100 μL of transformed cells were plated on selective LB 1.5% agar media supplemented with chloramphenicol (15 μg mL^{-1}) and incubated overnight at 37 °C.

2.4.7   Screening strategy

2.4.7.1   Screening yeast colonies

To identify positive clones generated using the PCR-based and TAR cloning methods, all individual yeast colonies were struck onto selective 2% agar plates containing synthetic complete media lacking histidine supplemented with adenine (60 mg L^{-1}) and grown overnight at 30 °C. Next, each streak was passed onto a second selective 2% agar plate and grown overnight at 30 °C. For TAR cloning, approximately 10 yeast colonies were pooled, to create 20 pools in total, by picking up a small amount of each colony and resuspending them together in 100 μL of TE buffer in 200 μL PCR tubes. For PCR-based cloning, the same protocol was followed except that cells from 20 individual colonies were used for each reaction. Subsequently, for both TAR cloning and PCR-based cloning methods, the resuspended cells were incubated at 95 °C for 15 min to lyse the cells. The tubes were then centrifuged for ~30 s using a mini centrifuge. Next, 1 μL of the supernatant was used as the DNA template for diagnostic MPX PCR.

MPX primer pairs were designed to have an optimal melting temperature of 60 °C using the online tool Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). MPX PCR was performed according to the Qiagen Multiplex PCR Handbook. To test potential pPT-TAR and pPT-PCR clones, two sets of MPX PCR were run, each yielding three amplicons. The first primer set, BK901, 904, and 906 F/R, generated amplicons of 265-, 334-, and 507-bp sizes, respectively. The second primer set, BK 902, 903, and 905 F/R, generated

171-, 224-, and 405-bp amplicons, respectively. Then, 2 μL of the PCR products were loaded onto a 2% agarose gel for electrophoresis and analyzed. Next, DNA was isolated as described in Section 2.4.2.2 from selected positive clones and transformed into *E. coli* as described in Section 2.4.6.

2.4.7.2   Screening *E. coli* colonies

First, a range of one to eight *E. coli* colonies transformed with DNA from positive yeast clones were screened using the same MPX PCR method as described in Section 2.4.7.1. The selected positive colonies were subsequently screened by diagnostic restriction enzyme digestion. To perform diagnostic restriction enzyme digestion, DNA was isolated from *E. coli* as described in Section 2.4.2.2, and the concentration of total isolated DNA was obtained using the DeNovix Inc. DS-11 FX+ Spectrophotometer. DNA preparations were ~5000 ng μL^{-1} before digestion. Digestion reactions were generated using 5 μL of DNA, 2 μL of NEBuffer 3.1 restriction buffer, 0.2 μL of SrfI, 0.2 μL of SacII, and 12.6 μL of water. Reaction mixtures were incubated at 37 °C for 60 min, and then 10 μL was loaded onto a 1% agarose gel for electrophoresis.

For further confirmation, the isolated pDNA was submitted to the CCIB DNA Core at Massachusetts General Hospital for whole plasmid sequencing and reference mapping. Sequences obtained were aligned with their respective references using the algorithm built into Geneious version 2020.0. Alignment disagreements were identified as mutations, and each mutation was individually analyzed to curate a list of mutations (Supplemental Table B-2).

2.4.8   Evaluation of growth phenotypes of host strains

2.4.8.1   *E. coli* growth in liquid media

E. coli strains harboring plasmids with cloned mitochondrial genomes pPT-PCR C1 and C2, pPT-TAR C1 and C2, and control plasmids pAGE3.0 and pPtGE31 (lacking a

mitochondrial genome) were inoculated and grown overnight in 5 mL of LB media supplemented with chloramphenicol (15 μg mL^{-1}) at 37 °C with shaking at 225 RPM. The saturated cultures were diluted 100-fold into 5 mL of the same media and grown for 2 h in 50-mL conical Falcon tubes under the same conditions. The cultures were placed on ice and diluted to an OD$_{600}$ of 0.1 in LB media supplemented with chloramphenicol (15 μg mL^{-1}; uninduced) or LB media supplemented with chloramphenicol (15 μg mL^{-1}) and arabinose (100 μg mL^{-1}; induced). In quadruplicate, 200 μL of each uninduced and induced culture was aliquoted into a 96-well plate. Once loaded, the 96-well plate was placed in a 96-well plate reader, Epoch 2. While in the plate reader, the strains were incubated at 37 °C with continuous, double-orbital shaking. OD$_{600}$ measurements were taken every 15 min for 5 h, for a total of 20 readings using Gen5 data analysis software version 3.08. This experiment was performed twice; therefore, eight measurements were obtained and averaged for each strain, and the standard error of the mean was calculated. The doubling time (td) of each strain was determined.

2.4.8.2 *S. cerevisiae* growth in liquid media

S. cerevisiae strains harboring pPT-PCR C1 and C2, pPT-TAR C1 and C2, and pAGE3.0 and pPtGE31 control plasmids (lacking a mitochondrial genome), were inoculated and grown overnight in 5 mL of synthetic complete yeast media lacking histidine supplemented with adenine (60 mg L^{-1}) at 30 °C with shaking at 225 RPM. The saturated cultures were diluted 100-fold into 5 mL of the same media and allowed to grow for 2 h in a 50 mL conical Falcon tube under the same conditions. The cultures were diluted to an OD$_{600}$ of 0.1 in the same media, and 200 μL of each culture was aliquoted into a 96-well plate in quadruplicate. Once loaded, the 96-well plate was placed in a 96-well plate reader, Epoch 2. While in the plate reader, the strains were incubated at 30 °C with continuous, double orbital shaking. OD$_{600}$ measurements were taken every 15 min for 12 h for a total of 49 readings using Gen5 data analysis software version 3.08. Four measurements were obtained and averaged for each strain, and the standard error of the mean was calculated. The td of each strain was determined.

2.4.8.3 *E. coli* and *S. cerevisiae* growth on solid media

E. coli and *S. cerevisiae* strains harboring pPT-PCR C1 and C2, pPT-TAR C1 and C2, and pAGE3.0 and pPtGE31 control plasmids (lacking a mitochondrial genome), were inoculated and grown overnight in LB media supplemented with chloramphenicol (15 µg mL^{-1}) at 37 °C, and synthetic complete yeast media lacking histidine supplemented with adenine (60 mg L^{-1}) at 30 °C, respectively. The saturated cultures were diluted 100-fold in their corresponding media and grown for 2 h. The cultures were diluted to an OD$_{600}$ of 0.1, which was used to generate a range of dilutions for *E. coli* and *S. cerevisiae*. The dilution series was plated in 5 µL aliquots onto their corresponding selection plates: 1.5% agar LB plates supplemented with chloramphenicol (15 µg mL^{-1}; uninduced) or with chloramphenicol (15 µg mL^{-1}) and arabinose (100 µg mL^{-1}; induced) for *E. coli*, and 2% agar plates containing synthetic complete yeast media lacking histidine supplemented with adenine (60 mg L^{-1}) for *S. cerevisiae*. Dilution plates for *E. coli* strains were grown overnight at 37 °C, and dilution plates for *S. cerevisiae* strains were grown for two days at 30 °C.

2.5 References

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Chapter 3

3 Cloning of *Thalassiosira pseudonana*'s mitochondrial genome in *Saccharomyces cerevisiae* and *Escherichia coli*

The work presented in this chapter is adapted from:

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3.1 Introduction

Recent advancements in DNA sequencing and synthesis resulted in the development of a powerful set of biotechnology tools that can help to address global challenges in food and water sustainability, medicine production, and eco-friendly energies. Many potential organisms are under investigation for desirable properties useful for biotechnology applications. One attractive candidate is *Thalassiosira pseudonana*. This model-centric diatom is naturally found in oceanic water and plays a significant role in global carbon cycling and combatting climate change [1,2]. In addition, its silica frustule encasement is suitable for nanotechnologies and drug delivery [3,4]. Due to the growing interest in *T. pseudonana*, its nuclear, mitochondrial, and plastid genomes were sequenced [5–7], enabling the development of genetic tools and DNA delivery methods, such as bacterial conjugation and microparticle bombardment [8,9]. Additional genetic tools for *T. pseudonana* include selectable markers [9,10], promoters [9], transformation vectors [9], inducible protein expression [9], RNA interference [11,12], and CRISPR/Cas9 [10,13,14]. Finally, methods for isolating *T. pseudonana*'s chloroplast and mitochondria have been developed, and proteomic data made available [15]. Most of the described genetic tools allow the engineering of *T. pseudonana*'s nuclear genome; however, engineering its organelle genomes is still undeveloped. There are several advantages to engineering organelle genomes, including polycistronic gene organization, the lack of transgene silencing, reduced positional gene expression effects, and the

compartmentalization of biosynthetic pathways, each of which simplifies engineering [16]. In preparation for exploiting these qualities, organelle genomes from multiple species have been cloned [17–23].

We demonstrated the cloning of the mitochondrial genome of *P. tricornutum*, a model diatom algae species, in baker's yeast, *S. cerevisiae*, and *E. coli* [23]. *S. cerevisiae* has proven to be an excellent host for cloning large DNA fragments or whole genomes [19,24–27], and it was also demonstrated that chromosomes up to ~500 kbp could be cloned in *E. coli* [28]. To test the versatility and robustness of this method when applied to other algal species, we selected *T. pseudonana* because of the unique characteristics of its mitochondrial genome. First, the *T. pseudonana* mitochondrial genome is compact (~44 kbp), harboring a relatively small repeat region (~5 kbp) compared to the repeat region of *P. tricornutum* (~35 kbp). Second, *T. pseudonana* has a lower G+C%-content mitochondrial genome (30%) than *P. tricornutum* (35%). Third, *T. pseudonana*'s mitochondrial genome uses an alternative genetic code, which substitutes a typical stop codon (UGA) for a tryptophan residue [6]. This alternative genetic code could be beneficial during the development of a whole-genome delivery method, as any engineered selection markers integrated into this genome would only function when delivered to the mitochondrial compartment, eliminating the need to screen against nuclear transformants [29].

Here, we report the successful cloning of *T. pseudonana*'s mitochondrial genome in yeast and demonstrate that it can also be propagated in *E. coli*. In the first iteration (Design 1), the mitochondrial genome was cloned in its entirety (~44 kbp; ~58 kbp including pPtGE31 plasmid backbone); in the second iteration (Design 2), ~3.8 kbp of the ~5 kbp repetitive region was excluded (~40 kbp; ~58 kbp including pAGE3.0 plasmid backbone). Growth experiments performed on yeast in liquid media revealed that yeast strains carrying plasmids with cloned mitochondrial genomes had a slightly increased growth rate; however, after 24 h, the yeast strains grew to the same (Design 1) or slightly lower (Design 2) end-point densities compared to control strains. When these genomes were propagated in *E. coli* on a low copy number plasmid, they had the same growth rate and end-point densities as the control strains. However, when grown with arabinose to

increase the copy number of these genomes, all samples grew to significantly lower end-point densities after 11.5 h. Also, analysis of plasmids containing mitochondrial genomes following propagation in *E. coli* over 60 generations showed that about 17% of *T. pseudonana* mitochondrial genomes were mutated compared to 0% identified for equivalent experiments conducted using the *P. tricornutum* mitochondrial genome. Finally, RNA sequencing performed on *E. coli* harboring either alga's mitochondrial genome found that gene expression can be detected for *T. pseudonana* and *P. tricornutum* mitochondrial genes.

3.2   Results

3.2.1   Cloning of *T. pseudonana*'s mitochondrial genome

Using a PCR-based approach, we cloned *T. pseudonana*'s mitochondrial genome in its entirety (Design 1: 57,641 bp; composed of a 43,827 bp mitochondrial genome, 11,742 bp pPtGE31 plasmid backbone, and 2072 bp *URA3* additional selection marker for *S. cerevisiae*) as well as a reduced version lacking 3.8 kbp of the ~5.0 kbp repetitive sequence (Design 2: 57,557 bp; composed of a 40,034 bp mitochondrial genome and a 17,523 bp pAGE3.0 plasmid backbone) (Figure 3-1). For Design 1, the complete genome was PCR-amplified in eight overlapping fragments from total *T. pseudonana* DNA. Four additional overlapping fragments were amplified, including an *S. cerevisiae* selection marker *URA3* (Fragment 5) and the pPtGE31 plasmid backbone (Fragments 10–12) [30], which contains all the genetic elements required for plasmid propagation in yeast and *E. coli*. In total, 12 DNA fragments were amplified (Figure 3-1C) and assembled following transformation and homologous recombination in *S. cerevisiae*, yielding 187 yeast colonies (Table 3-1). For Design 2, the genome was PCR-amplified in seven overlapping fragments that excluded a 3.8 kbp repeat region. The pAGE3.0 plasmid [31] backbone was amplified as two additional fragments (Fragments 7–8) to provide all the genetic elements required for propagation in yeast and *E. coli*. In total, nine DNA fragments were amplified (Figure 3-1D) and assembled in yeast, yielding 680 colonies (Table 3-1). For each design of the mitochondrial genome, two clones identified as correct in yeast by
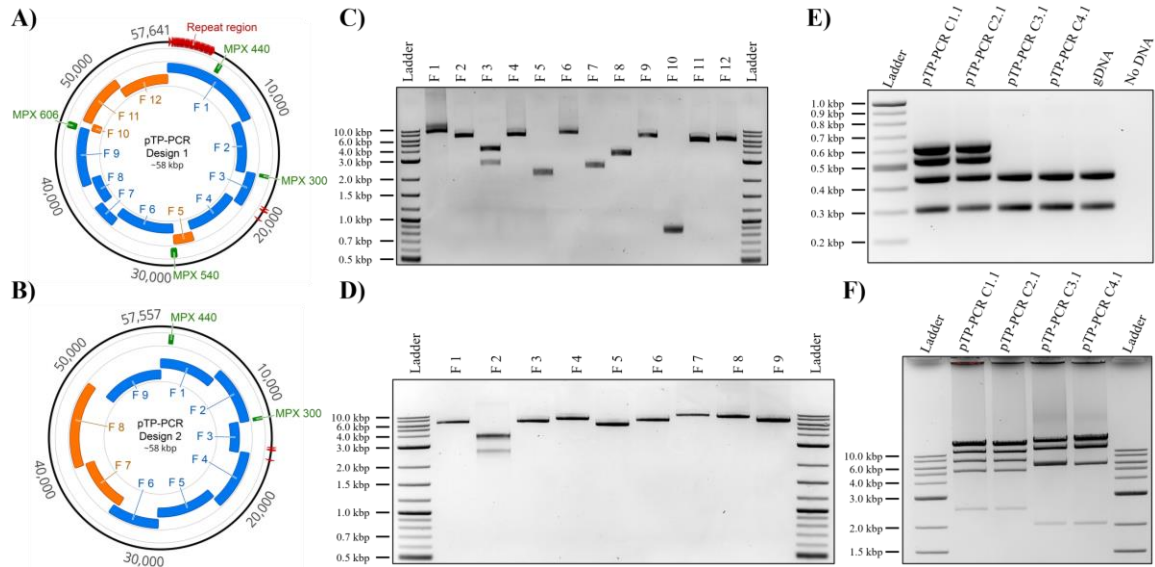
Figure 3-1. Design, amplification, and analysis of cloned *T. pseudonana* mitochondrial genomes. A, B) Plasmid maps of *T. pseudonana* mitochondrial genomes cloned with the repeat region (**A**—Design 1) or without (**B**—Design 2). The relative sizes and positions of the mitochondrial genome fragments (blue) and plasmid backbone fragments (orange) are shown. In addition, the four MPX PCR amplicons used for diagnostic screening and their sizes in bp are indicated (green). These images were generated using Geneious version 2020.2.4, created by Biomatters. **C)** Agarose gel electrophoresis of the 12 PCR-amplified fragments used to assemble plasmids as specified in Design 1. The resulting amplicon sizes for fragments 1–12 are 10,735-, 6092-, 3610-, 6274-, 2152-, 7035-, 2512-, 3250-, 6216-, 859-, 5367-, and 5870-bp, respectively. Note: Fragment 3 had a nonspecific amplicon but did not prevent the correct assembly. **D)** Agarose gel electrophoresis of the nine PCR-amplified fragments used to assemble plasmids as specified in Design 2. The resulting amplicon sizes for fragments 1–9 are 6092-, 3610-, 6254-, 7174-, 5417-, 6372-, 9136-, 8441-, and 6810-bp, respectively. Note: Fragment 2 had a nonspecific amplicon but did not prevent the correct assembly. **E)** MPX PCR screen of four cloned algal mitochondrial genomes isolated from *E. coli* with expected amplicon sizes of 300-, 440-, 540-, and 606-bp. Note: MPX amplicons 540- and 606-bp can only be amplified from Design 1 genomes. **F)** Diagnostic restriction digest of the four cloned algal mitochondrial genomes. For Design 1 genomes (pTP-PCR C1.1/2.1), after PvuI restriction enzyme digestion, the expected band sizes are 6-, 2454-, 4862-, 6262-, 12,903-, 15,405-, and 15,749-bp. For Design 2 genomes (pTP-PCR C3.1/4.1), after PmeI and BamHI restriction enzymes digestion, expected band sizes are 2031-, 5693-, 12,012-, 16,721-, and 20,960-bp.

Table 3-1. Cloning of the *T. pseudonana* full and reduced mitochondrial genomes in the host organisms *S. cerevisiae* and *E. coli*. Two PCR-based cloning assemblies were performed in *S. cerevisiae*. Correct genomes identified by MPX PCR were subsequently transformed into *E. coli*. The diagnostic MPX PCR was repeated on *E. coli* clones, and the final genomes were selected. For the *E. coli* media, CM indicates chloramphenicol antibiotic. For the *S. cerevisiae* selection media, -HIS indicates synthetic complete media lacking histidine. Four-amplicon MPX PCR, as shown in Figure 3-1E), was used.

Genome design Assembly type	*S. cerevisiae*			*E. coli*			
	Selection media	Colony count	MPX PCR screen	Selection media	Selected yeast colony: *E. coli* colony count	MPX PCR screen	Final genomes selected for analysis
1 – Full genome PCR – 12 fragments	-HIS -Uracil	187	15/20	CM	Yeast colony C1: 11 Yeast colony C2: 1137	C1 = 8/8 C2 = 4/4	pTP-PCR C1.1 pTP-PCR C2.1
2 – Reduced genome PCR – 9 fragments	-HIS	680	18/20	CM	Yeast colony C1: 4366 Yeast colony C2: 3530	C1 = 5/5 C2 = 5/5	pTP-PCR C3.1 pTP-PCR C4.1

diagnostic MPX PCR were selected and transformed into *E. coli*. After moving the assembled plasmids to *E. coli*, they were validated by diagnostic MPX PCR and restriction enzyme digest (Figure 3-1E, F). For Design 1, the two selected clones were named pTP-PCR C1.1/C2.1, and, for Design 2, pTP-PCR C3.1/C4.1. All four clones were sequenced and analyzed for mutations.

3.2.2 Sequence analysis of cloned *T. pseudonona* mitochondrial genomes

Sequences obtained for the pTP-PCR plasmids were aligned to reference sequences, and upon analyzing mutations, pTP-PCR C1.1, C2.1, C3.1, and C4.1 had an average of 18 mutations per mitochondrial genome (Table 3-2, Supplementary Table C-2). We observed approximately twice the number of mutations in clones for Design 1; however, most of these mutations mapped to the repetitive region (Supplementary Table C-2), which could be due to sequencing errors. Mutations could have also occurred during the cloning process (PCR amplification of fragments) or propagation in the host organisms. It is also plausible that some of these variants could naturally exist in the population of *T. pseudonana* mitochondrial genomes or be variations between our strain and the sequenced genome. If desired, individual fragments could be cloned and confirmed by sequencing before use in yeast assembly.

3.2.3 Maintenance of *T. pseudonona*'s mitochondrial genome in host organisms

We sought to examine the burden of propagating the cloned mitochondrial genomes in eukaryotic and prokaryotic host strains. *S. cerevisiae* and *E. coli* were used as host organisms to clone and store the *T. pseudonana* mitochondrial genome. We measured the growth of *E. coli* and *S. cerevisiae* strains in liquid media using a 96-well plate reader. Growth experiments performed for *S. cerevisiae* revealed that strains carrying plasmids with cloned mitochondrial genomes had a slightly increased growth rate; however, after

Table 3-2. Summary of mutations identified in the cloned *T. pesudonana* mitochondrial genomes. Identified mutations are categorized as point mutations (synonymous, missense, nonsense, and those found in non-coding regions) or gap mutations (insertions and deletions, either non-coding or coding).

Clone	Point mutations				Gap mutations				Total
					Non-coding		Coding		
	Synonymous	Missense	Nonsense	Non-coding	Insertion	Deletion	Insertion	Deletion	
pTP-PCR C1.1	1	6	0	8	0	6	0	3	24
pTP-PCR C2.1	1	3	0	7	2	8	0	2	23
pTP-PCR C3.1	0	5	0	0	0	1	2	4	12
pTP-PCR C4.1	1	5	0	3	0	1	0	2	12

24 h, the yeast strains grew to the same (Design 1) or slightly lower (Design 2) end-point densities as compared to control strains (Figure 3-2A and Figure 3-3A–D). For propagation in *E. coli*, we tested conditions where plasmids with mitochondrial genomes were maintained either as low or high (induced with arabinose) copy number. When these genomes were propagated in *E. coli* without arabinose induction to high plasmid copy number, there was no significant difference in the growth rate compared to the control strain (Figure 3-2B and Figure 3-3E, F). When grown with arabinose, all samples grew to a significantly lower end-point density than the uninduced strains (Figure 3-2B and Figure 3-3G, H); however, there were no significant differences between the control plasmid and plasmids harboring a mitochondrial genome within each growth condition (Figure 3-3G, H).

Additionally, when propagated in *E. coli* for an extended time (greater than 50 generations), we observed that a small fraction of genomes were mutated, as was evident by an absent PCR amplicon when clones were evaluated with MPX PCR (data not shown). To further investigate this, we evaluated one cloned mitochondrial genome (pTP-PCR C2.1) directly after transferring from yeast to *E. coli* ("G0") or after ~60 generations ("G60"). Since we did not observe similar mutations in our previous work cloning the *P. tricornutum* mitochondrial genome, we used our cloned *P. tricornutum* mitochondrial genome (pPT-TAR C1) as a control [23]. In total, 30 colonies for both *T. pseudonana* (from clone pTP-PCR C2.1) and *P. tricornutum* (from clone pPT-TAR C1) were evaluated at G0 and G60 using a higher resolution MPX PCR screen with six amplicons.

At G0, all 30 *E. coli* clones harboring either plasmid showed successful amplification of all six amplicons. At G60, all 30 *E. coli* clones harboring pPT-TAR C1 had a complete genome as analyzed by MPX PCR, suggesting that, over 60 generations, this plasmid is stably maintained. However, only 25 of 30 *E. coli* clones containing pTP-PCR C2.1 had complete genomes at G60, as analyzed by MPX PCR (Figure 3-4). These 5 clones were further analyzed by restriction enzyme digest (Supplementary Figure C-1). Four of these plasmids showed aberrant restriction enzyme banding patterns, suggesting a
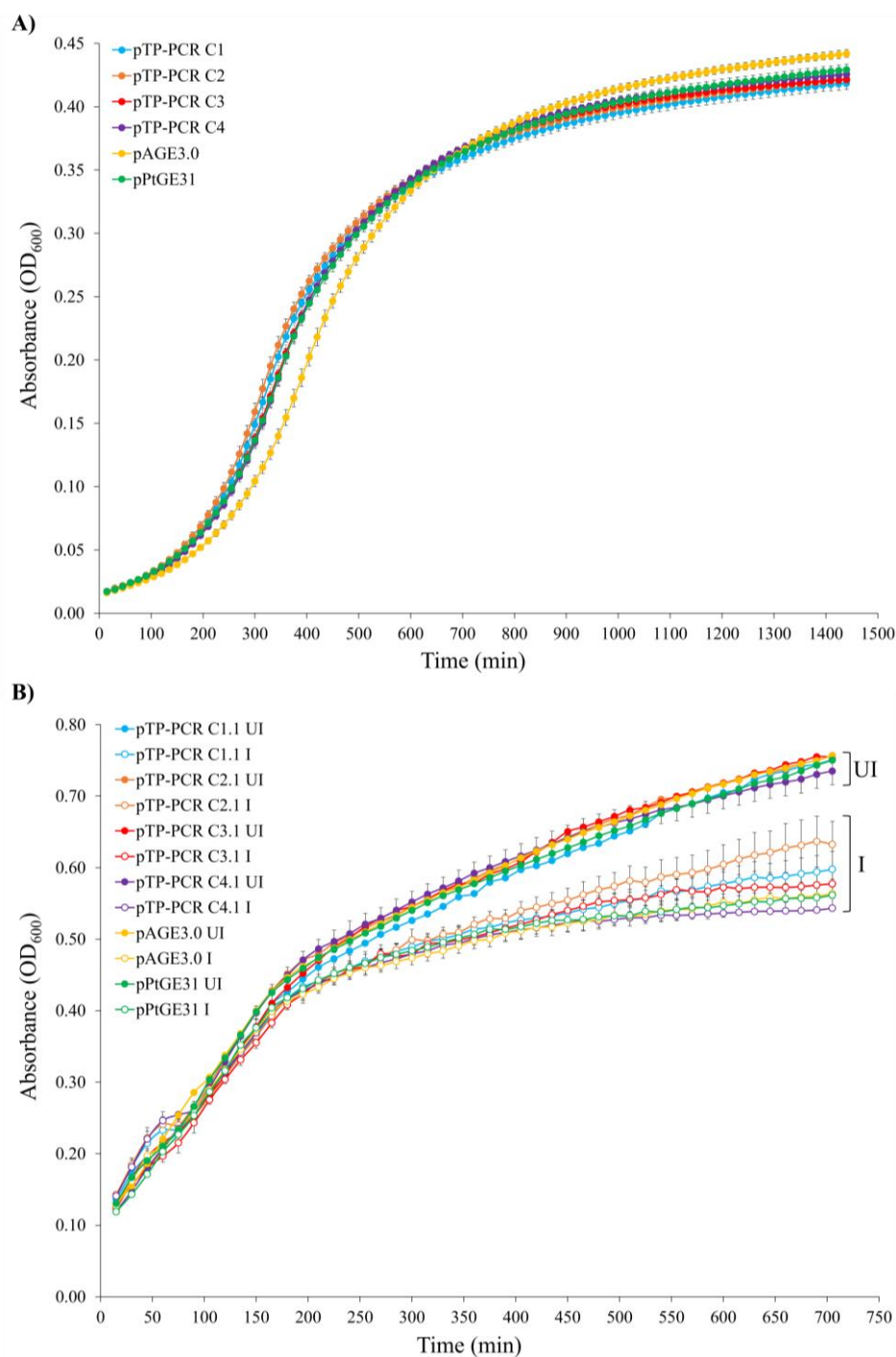
Figure 3-2. Growth of host strains harboring cloned *T. pseudonana* mitochondrial genomes in liquid media. A) Growth curves of *S. cerevisiae* strains grown in liquid synthetic complete media lacking histidine. **B)** Growth curves of *E. coli* strains grown in liquid LB media supplemented with chloramphenicol only (UI—un-induced) or with chloramphenicol and arabinose (I—induced). Each time point is the average of three biological replicates, each with four technical replicates and error bars representing the standard error of the mean.
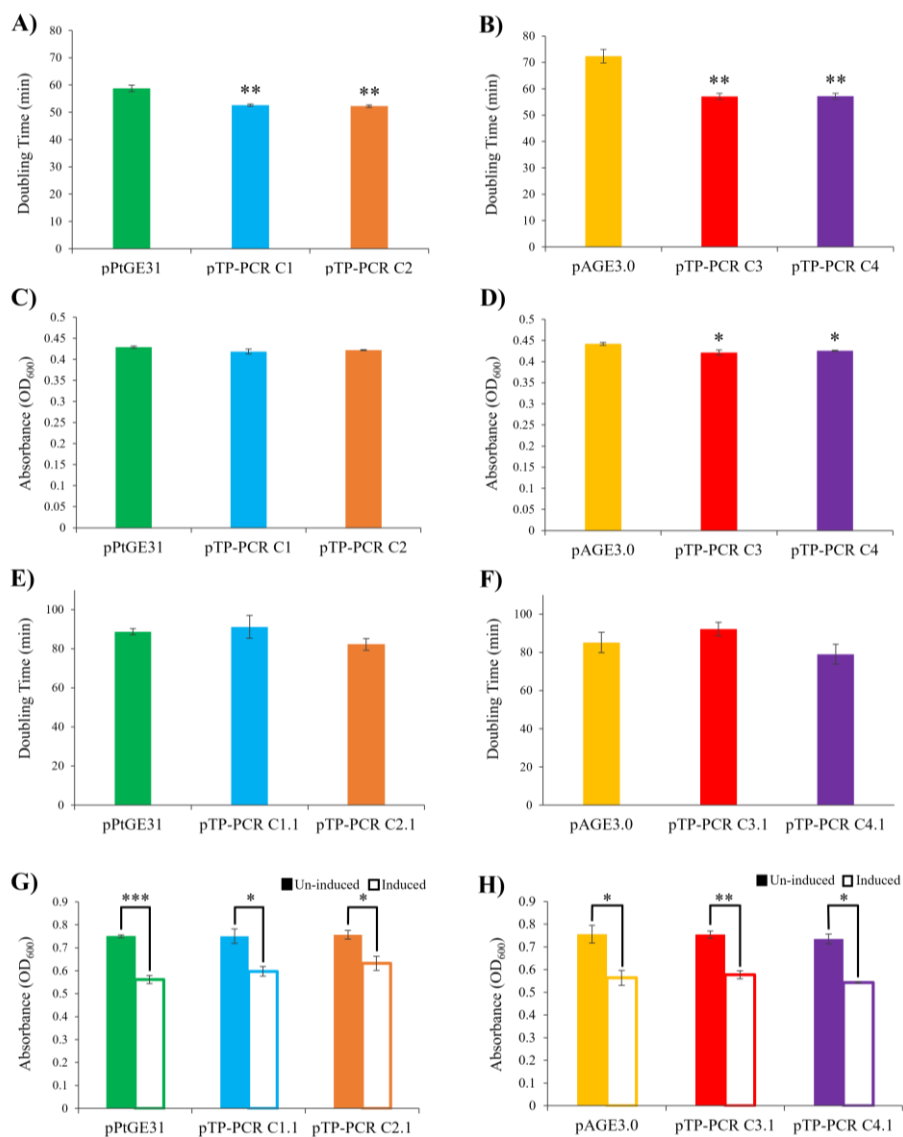
Figure 3-3. Growth phenotypes of *S. cerevisiae* and *E. coli* harboring a cloned *T. pseudonana* mitochondrial genome. A–B) The growth rate of *S. cerevisiae* harboring the full (**A**) and reduced (**B**) mitochondrial genome of *T. pseudonana* compared to control plasmids pPtGE31 and pAGE3.0, respectively. **C–D)** The maximum cell density reached by *S. cerevisiae* harboring the full (**C**) and reduced (**D**) mitochondrial genome compared to control plasmids. **E–F)** The growth rate of *E. coli* harboring the full (**E**) and reduced (**F**) mitochondrial genome, compared to control plasmids (uninduced conditions). **G–H)** Maximum cell density reached by *E. coli* harboring the full (**G**) and reduced (**H**) mitochondrial genome compared to control plasmids. Maximum density was compared in uninduced and arabinose-induced conditions. Note: Solid bars represent uninduced, and outlined bars represent induced conditions. Three biological replicates, each with four technical replicates, were used for data analysis. The scores represent means ± standard error of the mean. Asterisks represent a significant difference from control plasmid (**A–F**) and/or between uninduced and induced *E. coli* harboring the same plasmid (**G–H**) (Student's *t*-test: * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$).
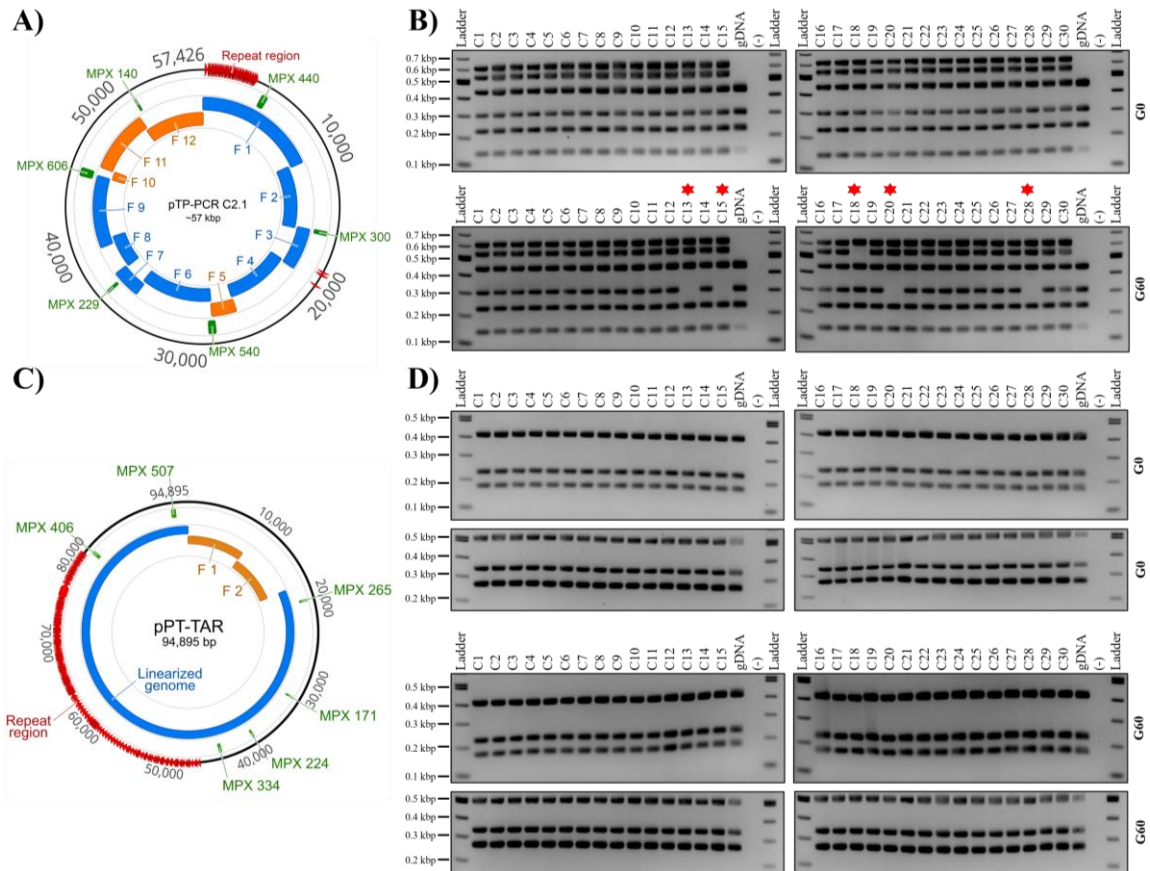
Figure 3-4. Plasmid stability assay of cloned *T. pseudonana* and *P. tricornutum* mitochondrial genomes over 60 generations. Thirty single colonies of either pTP-PCR C2.1 (**A, B**) or pPT-TAR C1 (**C, D**) were assayed by MPX PCR after transfer from yeast to *E. coli* (G0), and after ~60 generations (G60) in liquid LB media supplemented with chloramphenicol (15 μg mL^{-1}). Notes: 1—in **B)**, G60 colonies 13, 15, 18, 20, and 28 (red asterisk) are missing one PCR amplicon; 2—for *T. pseudonana* genomic DNA (gDNA), only the three fragments were expected to amplify, 229-, 300-, and 440-bp, but a small nonspecific band is also visible around 150 bp.

deletion or rearrangement. Three of these plasmids were sequenced, which confirmed that the absent MPX amplicon resulted from deletion events (Supplementary Figure C-2).

3.2.4   Assessing the expression of *T. pseudonona* and *P. tricornutum* mitochondrial genes in *E. coli*

RNA expression of mitochondrial genes was confirmed for pTP-PCR C2.1 and pPT-PCR C2.1 in *E. coli* with three biological replicates. Read counts were compared against pPtGE31 (lacking the mitochondrial genome). As expected, no reads from pPtGE31 mapped against the algal mitochondrial genomes, while genes from the pTP-PCR C2.1 and pPT-PCR C2.1 samples showed low read coverage. Low coverage of the mitochondrial genomes was likely obtained because the rRNA depletion kit selected failed to deplete the *E. coli* host's rRNA. The bacterial selection marker *cat* (providing resistance to chloramphenicol) was detected in all samples as expected; however, low read coverage was obtained for many of the genes on the pTP-PCR C2.1 and pPT-PCR C2.1 mitochondrial genomes (Supplementary Tables C-3 and C-4). The genes with the most mapped reads from the mitochondrial genome plasmids were rRNA and *cox1* genes. Although low read coverage was obtained, the counts confirm the expression of many of the mitochondrial genes from plasmids propagated in the *E. coli* hosts.

3.3   Discussion

The biotechnological potential of organelle engineering is constrained by the lack of reliable methods to clone and deliver organelle genomes to the corresponding compartment. Toward this goal, we developed a method for cloning and manipulating *P. tricornutum* mitochondrial genomes in host organisms [23]. Here, we demonstrated that this method could be adapted to another microalga, *T. pseudonana*. As for *P. tricornutum*, we observed similar growth rates between host strains carrying empty plasmids and those with cloned *T. pseudonana* mitochondrial genomes. RNA expression analysis showed that many of the genes from plasmids habouring *P. tricornutum* or *T.*

pseudonana's mitochondrial genome are expressed in *E. coli*. Interestingly, mitochondrial gene expression in the host did not affect genome stability for *P. tricornutum*'s mitochondrial genome. Plasmids with *T. pseudonana*'s mitochondrial genomes were less stable with continued propagation in *E. coli*; after 60 generations, 17% of genomes were mutated.

It has been observed previously that cloning low G+C%-content DNA into bacteria can be problematic. As the G+C%-content decreases, the probability of any sequence producing a spontaneous promoter or origin of replication becomes more likely, which can result in plasmid toxicity and instability [36]. The addition of a second origin of replication can stall the replication fork, leading to plasmid rearrangements [37]. Challenges arise with G+C%-contents as low as 35–40%; however, it was shown that this DNA could be stably maintained at a low copy number [36]. Additionally, it has been shown that by engineering the vector backbone to be more accommodating, low G+C%-content genomes such as *Lactobacillus helveticus* (35%) could be cloned [36]. A future investigation could focus on optimizing the pTP-PCR plasmids for stability.

Although we can confirm that most of the mitochondrial genes on the pTP-PCR C2.1 and pPT-PCR C2.1 plasmids are expressed in *E. coli* hosts, the low read coverage obtained prevented us from performing a reliable differential expression analysis. However, the data does demonstrate that genes of the algal mitochondrial genomes are expressed in the *E. coli* hosts. To determine what genes of the mitochondrial genomes are more strongly expressed in the *E. coli* hosts, we will need to obtain higher coverage of the mitochondrial genome by successfully depleting the host's rRNA in a future experiment.

Now that two algal mitochondrial genomes have been cloned in host strains, we better understand potential hurdles that can be encountered when applying this method to other species. As a next step, a robust method for delivering these genomes to mitochondria will need to be developed. First, the mitochondrial genomes must be engineered with mitochondria-specific selectable markers. Using antibiotics targeting organelle-specific processes, previous studies have demonstrated an increased efficacy of

antibiotic resistance proteins when localized to the organelle compartment [38,39]. Further, two promising antibiotic selection markers, zeocin and chloramphenicol have been described for use in the mitochondria of *Chlamydomonas reinhardtii* [40] and the chloroplast of *P. tricornutum* [41], respectively. Additionally, any selection markers generated for *T. pseudonana*'s mitochondria will be designed using an alternative genetic code (UGA will be translated as a tryptophan instead of a stop codon), allowing proper antibiotic-resistance proteins to be expressed only in the mitochondrial compartment. This design feature will generate a powerful system for developing the genetic tools required for mitochondrial DNA delivery in *T. pseudonana*.

3.4   Conclusions

We have demonstrated that a previously developed method for cloning and manipulating mitochondrial genomes can be applied to additional microalga. With a PCR-based approach, we cloned the mitochondrial genome of *T. pseudonana* in its entirety (~44 and; ~58 kbp including the pPtGE31 plasmid backbone) or lacking a repetitive region (~40; ~58 kbp including the pAGE3.0 plasmid backbone). The cloned genomes imposed no substantial growth burden on *S. cerevisiae* and *E. coli* when these host organisms were used to propagate the plasmids. In *E. coli*, some plasmid instability was observed after 60 generations, likely attributable to the low G+C%-content of the mitochondrial genome. RNA sequencing was performed, and it was found that mitochondrial genes were being expressed from the plasmids harbored in *E. coli*. In this study, we replicated the previous methods for cloning and manipulating algal mitochondrial genomes using *T. pseudonana*. Subsequent work should focus on developing the technologies required for efficient mitochondrial DNA delivery.

3.5   Materials and Methods

3.5.1   Strains and growth conditions

Thalassiosira pseudonana (Culture Collection of Algae and Protozoa CCAP 1085/12) was grown in L1 media supplemented with 200 μM of sodium silicate (Na$_2$SiO$_3 \cdot$9 H$_2$0) at 18 °C under cool white fluorescent lights (75 μE m^{-2} s^{-1}) and a photoperiod of 16 h light: 8 h dark. L1 media was made as previously described [8]. *Saccharomyces cerevisiae* VL6−48 (ATCC MYA-3666: *MATα*, *his3-Δ200*, *trp1-Δ1*, *ura3−52*, *lys2*, *ade2−101*, *met14*, psi$^+$, cir^0) was grown at 30 °C in rich yeast media (2 x YPDA), or synthetic complete media lacking either histidine or both uracil and histidine. After yeast spheroplast transformation, all synthetic complete media used contained 1 M sorbitol [27]. *Escherichia coli* (Epi300, Lucigen) was grown at 37 °C in either LB media alone or supplemented with chloramphenicol (15 μg mL^{-1}).

3.5.2   Total DNA isolation by modified alkaline lysis

DNA from *E. coli*, *S. cerevisiae*, and algae were isolated as previously described in Section 2.4.2.2.

3.5.3   DNA fragment preparation for PCR-based cloning

3.5.3.1   Design 1–full genome (pTP-PCR C1 and C2)

Mitochondrial genomes were cloned using the previously described method [23]. PCR amplification of mitochondrial fragments was performed using *T. pseudonana* total DNA. The mitochondrial genome was amplified as eight overlapping fragments (primers: P 1–4F/R and 6–8F/R, listed in Supplementary Table C-1), as well as four additional fragments (primers: P 5F/R and 10–12F/R, listed in Supplementary Table C-1) to amplify the *URA3* yeast selection marker and pPtGE31 plasmid backbone [30]. The pPtGE31 plasmid contains all the genetic elements required for selection and stable propagation in *S. cerevisiae*, *E. coli*, and *P. tricornutum*. In addition, this plasmid contains an *oriT* to allow for plasmid transfer using bacterial conjugation. All primers were manually

designed. Forward and reverse primers for fragments 2–3, 7–8, and the reverse primer of fragment 1 were designed to be 40 bp in length. Primers 60 bp in length were designed for fragments 4–6, 9–12, and the forward primer of fragment 1. Overlapping homology between fragments was between 80 and 635 bp to allow for efficient yeast assembly.

Each fragment was individually amplified in a 50 μL PCR reaction using 1 μL of PrimeSTAR GXL polymerase, 1 μL of template DNA (1–100 ng μL^{-1} isolated total DNA from either *T. pseudonana* or *E. coli*), and the respective forward and reverse primers each at a final concentration of 0.2 μM. The thermocycler conditions for fragments 2, 4–9, and 11–12 was as follows: 25 cycles of 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 600 s, and one cycle of 68 °C for 600 s, finishing with an infinite hold at 12 °C. The thermocycler for fragment 1 was programmed as follows: 5 cycles of 98 °C for 10 s, 50 °C for 15 s, and 68 °C for 420 s, followed by 20 cycles of 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 420 s, and one cycle of 68 °C for 600 s, finishing with an infinite hold at 12 °C. The thermocycler for fragment 3 was programmed as follows: 25 cycles of 98 °C for 10 s, 50 °C for 15 s, and 68 °C for 660 s, and one cycle of 68 °C for 600 s, finishing with an infinite hold at 12 °C. The thermocycler for fragment 10 was programmed as follows: 25 cycles of 98 °C for 10 s, 55 °C for 15 s, and 68 °C for 60 s, and one cycle of 68 °C for 600 s, finishing with an infinite hold at 12 °C. PCR product amplification was confirmed by performing agarose gel electrophoresis with 2 μL of PCR product on a 1.4% agarose (*w/v*) gel. To eliminate plasmid template DNA, PCR products were treated with DpnI restriction enzyme as previously described in Section 2.4.3.

3.5.3.2   Design 2–reduced genome lacking repetitive region (pTP-PCR C3 and C4)

PCR amplification of mitochondrial fragments was performed using isolated *T. pseudonana* total DNA as the template DNA. The mitochondrial genome was amplified as seven overlapping fragments (primers: P 1R, 2–3F/R, 4F, 6R, 8R, 13–14F/R, and 17–18F, listed in Supplementary Table C-1), as well as two additional fragments (primers: P15–16F/R, listed in Supplementary Table C-1) to amplify the pAGE3.0 plasmid with

homology to sequence directly flanking the repeat region. The pAGE3.0 plasmid is a derivation of pPtGE31 providing additional elements for selection and stable propagation in *Sinorhizobium meliloti* [31]. All primers were manually designed. Forward and reverse primers for fragments 1–2, 5, the forward primer for fragments 4 and 6, and the reverse primer for fragments 3 and 9 were designed to be 40 bp long. Primers 60 bp in length were designed for the forward primer of fragment 3 and the reverse primer of fragment 4. The reverse primer of fragment 6, forward primer of fragment 9, and primers of fragments 7–8 were 80 bp in length. Overlapping homology between fragments was between 80 and 635 bp to allow for efficient yeast assembly.

Each fragment was individually amplified in a 50 µL PCR reaction using 1 µL of PrimeSTAR GXL polymerase, 1 µL of template DNA (1–100 ng µL^{-1} isolated total DNA from either *T. pseudonana* or *E. coli*), and the respective forward and reverse primers each at a final concentration of 0.2 µM. The thermocycler for fragments 1, 3–5, and 7–9 was programmed as follows: 25 cycles of 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 600 s, and one cycle of 68 °C for 600 s, finishing with an infinite hold at 12 °C. The thermocycler for fragments 2 and 6 was programmed as follows: 30 cycles of 98 °C for 10 s, 50 °C for 15 s, and 68 °C for 660 s, followed by one cycle of 68 °C for 600 s, finishing with an infinite hold at 12 °C. PCR product amplification was confirmed by performing agarose gel electrophoresis with 2 µL of PCR product on a 1.4% agarose (*w/v*) gel. To eliminate plasmid template DNA, PCR products were treated with DpnI restriction enzyme as described previously in Section 2.4.3.

3.5.4   Yeast spheroplast transformation protocol

Yeast spheroplast transformation was performed as previously described in Section 2.4.5.

3.5.5   *E. coli* transformation

E. coli transformation was performed as previously described in Section 2.4.6.

3.5.6   Screening strategy

3.5.6.1   Screening Yeast Colonies

To identify positive clones, individual yeast colonies were screened as previously described in Section 2.4.7.1, using selective 2% agar plates containing synthetic complete media lacking either histidine and uracil for pTP-PCR C1/2 or histidine for pTP-PCR C3/4. MPX primers used for screening yeast colonies differ from the original protocol and are listed in Supplementary Table C-1.

3.5.6.2   Screening *E. coli* Colonies

To identify positive clones, individual *E. coli* colonies were screened as previously described in Section 2.4.7.2 with the following modifications to the restriction enzyme digestion reactions. For pTP-PCR C1.1/2.1, digestion reactions were generated using 7 μL of DNA (~5000 ng μL^{-1}), 2 μL of NEBuffer 3.1 restriction buffer, 0.4 μL PvuI, and 10.6 μL of water. For pTP-PCR C3.1/4.1, digestion reactions were generated using 7 μL of DNA (~5000 ng μL^{-1}), 2 μL of CutSmart Buffer, 0.4 μL PmeI, 0.4 μL BamHI-HF, and 10.2 μL of water. Reaction mixtures were transferred to a Bio-Rad thermocycler and incubated either at 37 °C for 90 min or 37 °C for 90 min, followed by 65 °C for 20 min for pTP-PCR C1.1/2.1 and C3.1/4.1, respectively. After confirmation by MPX PCR and diagnostic restriction enzyme digestion, the four plasmids underwent next-generation whole plasmid sequencing at CCIB DNA Core at Massachusetts General Hospital.

3.5.7   Evaluation of growth phenotypes of host strains

3.5.7.1   *S. cerevisiae* growth in liquid media

Growth rates were evaluated for *S. cerevisiae* strains harboring pTP-PCR C1, C2, C3, and C4 plasmids, or pPtGE31 and pAGE3.0 control plasmids (lacking a mitochondrial genome), as previously described in Section 2.4.8.2, with absorbance (OD$_{600}$) measurements taken every 15 min for 24 h. This experiment was performed with three

biological replicates, each with four technical replicates; therefore, 12 readings were obtained and averaged for each strain, the standard error of the mean was calculated, and the curves were plotted. End-point densities at 1440 min were averaged for each strain, and the standard error of the mean was calculated. The td of each replicate was determined using the R package Growthcurver (http://github.com/sprouffske/growthcurver) [32]. The td was averaged from the 12 replicates for each strain, and the standard error of the mean was calculated.

3.5.7.2   *E. coli* growth in liquid media

Growth rates were evaluated for *E. coli* strains harboring pTP-PCR C1.1, C2.1, C3.1, and C4.1 plasmids or pPtGE31 and pAGE3.0 control plasmids (lacking a mitochondrial genome), as previously described in Section 2.4.8.1, except the samples were not placed on ice and absorbance (OD_{600}) measurements were taken every 15 min for 11.5 h. This experiment was performed with three biological replicates, each with four technical replicates; therefore, 12 readings were obtained and averaged for each strain, the standard error of the mean was calculated, and the curves were plotted. End-point densities at 705 min were averaged for each strain, and the standard error of the mean was calculated. The td of each replicate was determined using the R package Growthcurver (http://github.com/sprouffske/growthcurver) [32]. The td was averaged from the 12 replicates for each strain, and the standard error of the mean was calculated. Replicates shown to be major outliers were removed from the dataset (Supplementary Note C-1).

3.5.8   Bacterial RNA extraction

E. coli harboring the pTP-PCR C2.1, pPT-PCR C2.1 [23], or pPtGE31 plasmid (lacking a mitochondrial genome) were inoculated overnight in LB media supplemented with chloramphenicol (15 μg mL^{-1}) from frozen strain stocks. In the morning, 1 mL of cells was diluted into 25 mL of LB media supplemented with chloramphenicol (15 μg mL^{-1}) and grown for 120 min at 37 °C until absorbance (OD_{600}) reached 1. Subsequently, the

RNA stabilization of the culture was performed using RNAprotect Bacteria Reagent. Briefly, 400 μL (2 x 10^8 cells) of culture was transferred to a 15 mL Falcon tube containing 800 μL of RNAprotect Bacteria Reagent, and the suspension was vortexed for 5 s and incubated at RT for 5 min. Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's instructions. Following treatment with DNase using RNase-free DNase Set, the RNA concentration was determined using DeNovix, and the integrity was verified by running 400 ng of RNA on a 1% agarose (*w/v*) gel. RNA samples were stored at -80 °C until use.

3.5.9   RNA sequencing

The quality of isolated RNA (Section 3.5.8) was validated using the Agilent Bioanalyzer 2100. The RNA library was created and sequenced using the NextSeq 550 platform (single end 150 mid-output), with rRNA depleted using the NEB bacterial rRNA depletion kit. Read quality was evaluated using FastQC v0.11.9, and the reads were trimmed using Trimmomatic v0.39 in single-end mode using the parameters AVGQUAL:25 CROP:150 MINLEN:100 [33–35]. Reads for the pTP-PCR C2.1 and pPT-PCR C2.1 strains were mapped against their respective mitochondrial plasmid maps using bowtie 2.26 in single-end mode with the parameters no-unal -k 1. Read counts were generated using htseq-count.

3.5.10   Plasmid stability assay

3.5.10.1   Propagation of *E. coli* strains

E. coli harboring either pTP-PCR C2.1 or pPT-TAR C1 [23] plasmids were inoculated from frozen strain stocks (note that these stocks were generated by transferring cloned mitochondrial DNA from yeast to *E. coli*, isolating single colonies on LB plates, and then grown overnight in liquid before freezing) overnight at 37 °C (225 RPM) in 5 mL of LB media supplemented with chloramphenicol (15 μg mL^{-1}). The saturated overnight culture

was adjusted to an OD_{600} of 0.1, and frozen strain stocks were generated (G0). Next, 1 µL of the adjusted culture was added to 50 mL of LB media supplemented with chloramphenicol (15 µg mL^{-1}). Cultures were grown at 37 °C (225 RPM) to an OD_{600} of ~3. Four repetitions (~60 generations) of diluting grown cultures to an OD_{600} of 0.1 and passaging 1 µL of adjusted culture into 50 mL of LB media supplemented with chloramphenicol (15 µg mL^{-1}) were performed. After four serial passages, frozen strain stocks of each bacterial strain adjusted to an OD_{600} of 0.1 were generated to analyze plasmid stability (G60).

3.5.10.2 Analysis of descendant *E. coli* colonies

Strain stocks of pTP-PCR C2.1 or pPT-TAR C1 from the start (G0) and end (G60) of propagation were thawed on ice for 20 min and then diluted 1:5000 with LB media in 1.5 mL microcentrifuge tubes. Next, 100 µL of each diluted culture was plated separately onto selective LB agar plates supplemented with chloramphenicol (15 µg mL^{-1}) and grown at 37 °C for 24 h to obtain single colonies. Thirty single colonies of each construct were struck onto selective LB agar plates supplemented with chloramphenicol (15 µg mL^{-1}) and grown for 12 h at 37 °C. Next, each streak was resuspended in a PCR tube containing 100 µL of TE buffer. The cell mixture was transferred to a Bio-Rad thermocycler and incubated at 95 °C for 15 min to lyse the cells. The cellular debris was pelleted to the bottom of the PCR tube using a microcentrifuge, and 1 µL of supernatant was used as a template for diagnostic MPX PCR. Two additional diagnostic primer sets (P 19–20F/R, listed in Supplementary Table C-1) were generated for this plasmid stability analysis. Primer pairs P 19–20F/R were designed to have an optimized melting temperature of 60 °C using the online tool Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). To test plasmid stability, primer sets P 19–24F/R generated 140-, 229-, 300-, 440-, 540-, and 606-bp amplicons, respectively. Then, 2 µL of the PCR products were loaded onto a 2% agarose (*w/v*) gel for electrophoresis and analyzed.

3.5.11   Statistical analyses

Statistical analyses were performed using Microsoft Excel spreadsheet software. Pair-wise comparisons were made using a Student's *t*-test with either equal or unequal variance based on the result of an F-test. The error bar shown represents the standard error of the mean.

3.6   References

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Chapter 4

4 Superior conjugative plasmids delivered by bacteria to diverse fungi

The work presented in this chapter is adapted from:

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4.1 Introduction

The fungal kingdom is exquisitely diverse and home to countless species with profound impacts on ecological nutrient cycling, industrial manufacturing, and health and disease in humans, animals, and plants [1,2]. Yeast species are amongst the best-studied fungi and include the common yeast *S. cerevisiae*, which is a primary fermenter of beer, wine, and bread, and a ubiquitous eukaryotic model system. *S. cerevisiae* is also an important synthetic biology chassis for producing insulin, vaccine components, and other critical recombinant proteins [3]. The closely related *Saccharomyces boulardii* is a promising probiotic therapeutic, particularly in the context of obesity and type 2 diabetes [4,5]. Yeasts are also critical components of the human microbiota, including *Candida* species associated with vaginal yeast infections and invasive candidiasis [6], and *Malassezia* species with notable associations with Crohn's disease and pancreatic cancer [7, 8]. The skin-associated yeast *Candida auris* is an emerging fungal pathogen that can cause life-threatening infections and is highly refractory to antifungal drug treatment [9–11]. These diverse and critical roles in health, disease, and industrial manufacturing highlight the importance of studying and manipulating the biology of these key yeast species.

Given the diversity of yeast species and the breadth of niches they inhabit, there is a need to develop improved and innovative methods for DNA transformation in these

organisms. Genetic transformation techniques enable the manipulation of genomes of industrially important yeasts and further promote the ability to target, modify, or damage the genomes of fungal pathogens. Indeed, genetic-editing tools such as CRISPR have a promising role as novel antimicrobial agents due to their ability to specifically target pathogen-associated genes, leading to microbial death, growth inhibition, or targeted deletion of genes involved in antimicrobial resistance or virulence [12–18]. However, laboratory-based transformation protocols typically rely on chemical strategies to promote DNA uptake, which is not broadly applicable for manipulating yeasts in their native environments, such as those inhabiting the microbiome. One innovative strategy to promote the uptake of genetic material *in situ* is to exploit bacterial conjugation as a viable mechanism to transfer plasmids from bacteria to a recipient microbe via the bacterial type IV secretion system. Previous work has demonstrated the utility of bacterial conjugation for transferring plasmids, including those encoding CRISPR-based antimicrobials, between bacterial species, both *in vitro* [19,20] and *in vivo* in mouse microbiome models [21–23]. While bacterial conjugation typically occurs between bacterial species, cross-kingdom bacterial conjugation from bacteria to yeast and algae has been demonstrated [24–27]. Despite recently optimized protocols [28, 29], bacterial conjugation to yeast still suffers from a relatively low bacterial conjugation efficiency compared to prokaryotic recipients.

Thus, we sought to improve DNA transfer from bacteria to yeast by optimizing the genetic bacterial conjugation machinery of the pTA-Mob 2.0 plasmid [28]. This conjugative plasmid is composed of genetic elements required for plasmid maintenance and transfer [30,31]. Two regions, Tra1 and Tra2, are responsible for the transfer of plasmid DNA. Tra1 harbors the relaxase (TraH–J), primase (TraA–G), and leader (TraK–M) operons, which together coordinate the mobilization of the plasmid to the recipient [31]. The relaxase and leader operons encode the relaxosome, a protein complex essential for initial DNA processing during bacterial conjugation. Assembly of the protein complex (TraH–J) is initiated by TraJ binding to the 19-bp inverted repeat sequence in the *oriT* [32–34]. The interaction of TraI and TraJ, which TraH stabilizes, then orients the relaxase toward the nick-site [34]. After the formation of the relaxosome, TraI nicks and

covalently binds to the pDNA, ready for transfer to the recipient cell [35,36]. The binding of TraK to the *oriT* orients the pDNA into a more favorable position increasing the nicking reaction's efficiency [33,37]. TraC1, of the primase operon, is a DNA primase that co-transfers (along with single-stranded binding proteins) with the DNA to the recipient cell, where it is involved in restoring a double-stranded plasmid [38]. The primase operon also includes the TraG protein, which couples DNA processing by the relaxosome to DNA transfer by delivering the protein-DNA complex to the mating pair formation proteins [39,40]. The Tra2 region contains proteins (TrbB–L and TraF) required for mating pair formation, many of which are associated with the cell membrane. TrbC encodes a peptide responsible for forming the pilus. This peptide undergoes maturation by proteolytic cleavage followed by cyclization by TraF, resulting in rigid pili [41,42]. The pilus allows initial contact between the two cells and enables the transfer of single-stranded pDNA to the recipient cell.

Here, we developed and validated novel plasmids for improved bacterial conjugation efficiency between bacteria and diverse yeast species. We demonstrated that a cluster mutation in the relaxase operon, specifically in the *traJ* promoter, significantly improved DNA transfer from bacteria to *S. cerevisiae* and diverse yeasts, including the emerging pathogen *C. auris*. We generated improved, streamlined, and Golden Gate (GG) assembly-compatible plasmid derivatives of pTA-Mob 2.0 to enable facile insertion of custom genetic cassettes. Finally, we demonstrated that these designer conjugative plasmids can be used as a novel antifungal reagent with important applications for developing next-generation antifungal therapeutics.

4.2   Results

4.2.1   Development of streamlined conjugation plasmids

As a first step (Figure 4-1) toward creating an optimized and minimized conjugative plasmid for yeast, 55 single genes or small genetic regions were individually deleted from our previously established trans-kingdom conjugation plasmid, pTA-Mob 2.0 [28]

Step 1. Created single gene/fragment deletions of conjugative plasmid pTA-Mob 2.0. Tested for conjugation to *S. cerevisiae* and classified as essential, semi-essential, or non-essential.

Step 2. Created cluster gene deletions of conjugative plasmid pTA-Mob 2.0 based on data from Step 1. Plasmids created: M1–M8.

Step 3. Tested conjugation of M1–M8 to *S. cerevisiae*. Plasmid M3 clone 1 showed significantly improved conjugation. Genetic changes responsible for improved conjugation were identified.

Step 4. Created superior conjugative plasmid pSuperCon5 (pSC5) based on data from Step 3. Additional features added to pSC5: antibiotic selection markers for diverse yeasts and diatoms.

Step 5. Demonstrated that pSC5 has improved conjugation to *S. cerevisiae* in *cis* and *trans* set up. The improved conjugation was reproduced from both donor strains: *E. coli* and *S. meliloti.*

Step 6. Demonstrated that pSC5 can be delivered to diverse yeast: *Candida* and *Metschnikowia* species.

Step 7. Domesticated pSC5 to allow for Golden Gate (GG) assembly.

New plasmid created: pSC5GGv1 or pSC5GGv2. As the first demonstration of GG assembly, an additional yeast antibiotic marker was added.

Step 8. Demonstration that delivery of designer pSC5 plasmids by conjugation can act as an antifungal.

Figure 4-1. Experimental Design. The eight-step flowchart shows experiments and major findings described in this article.

(Supplemental Figure D-1, Supplemental Table D-1). To validate these plasmid variants, up to two clones of each were tested for bacterial conjugation from *E. coli* to *S. cerevisiae*, and the deletions were classified as essential (no bacterial conjugation), semi-essential (decreased bacterial conjugation), or non-essential (near wild-type bacterial conjugation) for bacteria-to-yeast conjugation (Supplemental Table D-3). Based on this data, four streamlined plasmids were created where clusters of non-essential genes were simultaneously removed (plasmids M1–M4, Table 4-1, Supplemental Table D-2). Plasmids M1–M4 were then conjugated from *E. coli* to *S. cerevisiae*, and we observed a significant increase in bacterial conjugation efficiency for plasmid M3 clone 1 (M3C1), monitored by yeast colony formation on selective media (Figure 4-2). Sequencing both M3 clones, M3C1 and M3C2, revealed multiple mutations in each clone, which are likely responsible for the increase in bacterial conjugation efficiency for M3C1 (Supplemental Table D-4).

To identify which mutations in M3C1 were responsible for the increased bacterial conjugation efficiency, we performed a fragment-swapping experiment between M3C1 and M3C2 to produce five hybrid plasmids M3C1_F1–F5 (Figure 4-3). Each hybrid plasmid was created from four fragments amplified from M3C2 and one fragment from M3C1. The hybrid plasmid M3C1_F4 (fragment 4 originated from M3C1) had the closest bacterial conjugation phenotype compared to M3C1 (Figure 4-3). There were two mutated regions in fragment 4 of M3C1: a cluster of mutations in the promoter of *traJ* and a single mutation in the open reading frame (ORF) of *traJ* (Supplemental Table D-4). To validate which mutation(s) contributed to the increased conjugative phenotype, the promoter or ORF *traJ* mutations were each individually introduced into pTA-Mob 2.0 and tested for relative bacterial conjugation phenotypes. Only the mutations in the promoter region of *traJ* improved bacterial conjugation to yeast (Figure 4-3). Additionally, we continued to minimize M3C1 by creating new plasmids with additional non-essential genes removed to obtain the plasmids M5–M8 (Figure 4-2). Each minimized plasmid of M5–M8 still produced more colonies when conjugated to yeast relative to the original pTA-Mob 2.0 plasmid (Figure 4-2).

Table 4-1. Plasmids used in this study. *aacC1* provides resistance to gentamicin; *cat* to chloramphenicol; *bla* to ampicillin; y-*nat* to nourseothricin in diverse yeast; *Sh ble* to zeocin in diverse yeasts. *HIS3* is required for the histidine biosynthesis; *URA3* for uracil biosynthesis; *TRP1* for tryptophan biosynthesis in *S. cerevisiae*. [†] - duplicated copy that is present in the plasmid backbone.

Plasmid	Plasmid Size (kbp)	*E. coli* Marker	Yeast Marker	Citation
pTA-Mob 2.0	57	*aacC1*	*HIS3/URA3*	Soltysiak *et al.*, 2019
pAGE1.0	18	*cat*	*HIS3*	Brumwell *et al.*, 2019
pAGE2.0.T	19	*cat*	*TRP1*	This Study
pAGE2.0-i	20	*cat*	*HIS3*	This Study
pAGE2.0-iTraJ	20	*cat*	*HIS3*	This Study
pRS32	11	*bla*	*Sh ble*	Shapiro *et al.* (unpublished)
M1C1: pTA-Mob 2.0 Δ*trbO–fiwA*	54	*aacC1*	*HIS3/URA3*	This Study
M1C2: pTA-Mob 2.0 Δ*trbO–fiwA*	54	*aacC1*	*HIS3/URA3*	This Study
M2C5: pTA-Mob 2.0 Δ*klaC–kleA*	53	*aacC1*	*HIS3/URA3*	This Study
M3C1: pTA-Mob 2.0 Δ*istA–traB*	52	*aacC1*	*HIS3*	This Study
M3C2: pTA-Mob 2.0 Δ*istA–traB*	52	*aacC1*	*HIS3*	This Study
M4C16: pTA-Mob 2.0 Δ*istA–traE*	46	*aacC1*	*HIS3*	This Study
M4C19: pTA-Mob 2.0 Δ*istA–traE*	46	*aacC1*	*HIS3*	This Study
M5C1: pTA-Mob 2.0 Δ*trbN–traE*	40	*aacC1*	*HIS3*	This Study
M5C3: pTA-Mob 2.0 Δ*trbN–traE*	40	*aacC1*	*HIS3*	This Study
M6C2: pTA-Mob 2.0 Δ*trbN–traE*, Δ*klaC–kleA*	35	*aacC1*	*HIS3*	This Study
M6C4: pTA-Mob 2.0 Δ*trbN–traE*, Δ*klaC–kleA*	35	*aacC1*	*HIS3*	This Study
M7C2: pTA-Mob 2.0 Δ*trbN–traE*, Δ*trfA–traJ*[†]	37	*aacC1*	*HIS3*	This Study
M8C8: pTA-Mob 2.0 Δ*trbM–traE*, Δ*klaC–kleA*, Δ*trfA–traJ*[†]	31	*aacC1*	*HIS3*	This Study
M8C10: pTA-Mob 2.0 Δ*trbM–traE*, Δ*klaC–kleA*, Δ*trfA–traJ*[†]	31	*aacC1*	*HIS3*	This Study
M3C1_F1: pTA-Mob 2.0 Δ*istA–traB*	52	*aacC1*	*HIS3*	This Study
M3C1_F2: pTA-Mob 2.0 Δ*istA–traB*	52	*aacC1*	*HIS3*	This Study
M3C1_F3: pTA-Mob 2.0 Δ*istA–traB*	52	*aacC1*	*HIS3*	This Study
M3C1_F4: pTA-Mob 2.0 Δ*istA–traB*	52	*aacC1*	*HIS3*	This Study
M3C1_F5: pTA-Mob 2.0 Δ*istA–traB*	52	*aacC1*	*HIS3*	This Study
pTA-Mob 2.0 Tp	56	*aacC1*	*HIS3/URA3*	This Study
pTA-Mob 2.0 To	56	*aacC1*	*HIS3/URA3*	This Study
pSC5: pTA-Mob 2.0 Δ*istA–traB*	56	*aacC1*	*HIS3*/y-*nat*	This Study
pTA-Mob 2.1: pTA-Mob 2.0 Δ*traJ*[†]	56	*aacC1*	*HIS3/URA3*	This Study
pSC5.1: pTA-Mob 2.0 Δ*istA–traB*, Δ*traJ*[†]	55	*aacC1*	*HIS3*/y-*nat*	This Study
pSC5GGv1: pTA-Mob 2.0 Δ*istA–traB*, +*mrfp*	57	*aacC1*	*HIS3*/y-*nat*	This Study
pSC5GGv2: pTA-Mob 2.0 Δ*istA–traB*, +*mrfp*	57	*aacC1*	*HIS3*/y-*nat*	This Study
pSC5GGv1_ShBle: pTA-Mob 2.0 Δ*istA–traB*	57	*aacC1*	*HIS3*/y-*nat/Sh ble*	This Study
pSC5-toxic1: pTA-Mob 2.0 Δ*istA–traB*, +ACL0117	59	*aacC1*	*HIS3*/y-*nat*	This Study
pSC5-toxic2: pTA-Mob 2.0 Δ*istA–traB*, +HindII	57	*aacC1*	*HIS3*/y-*nat*	This Study
pSC5-toxic3: pTA-Mob 2.0 Δ*istA–traB*, +HindII	57	*aacC1*	*HIS3*/y-*nat*	This Study
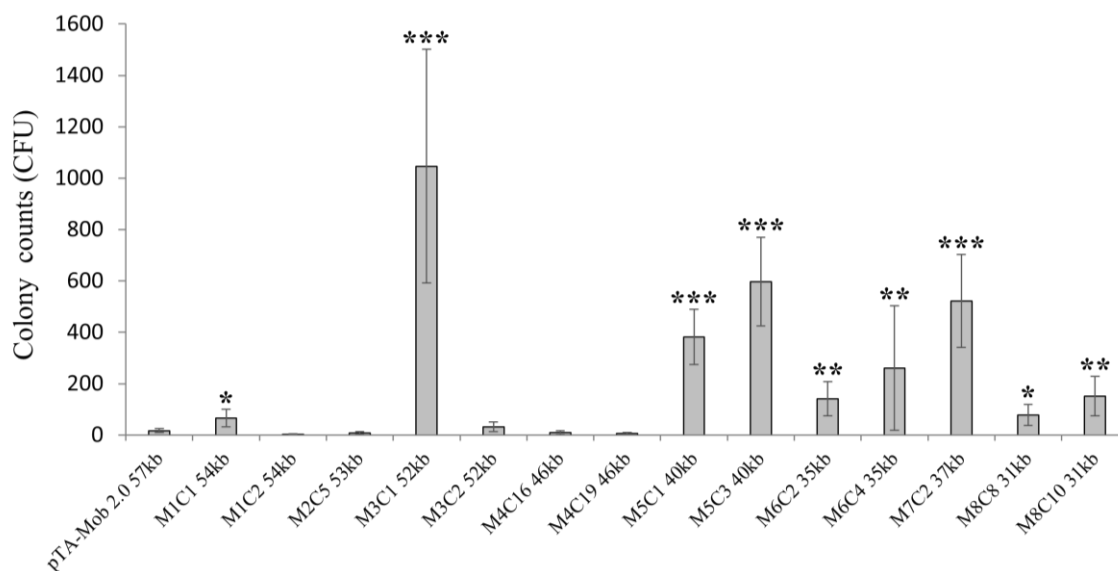
Figure 4-2. Transfer of minimized plasmids from *E. coli* to *S. cerevisiae* via bacterial conjugation. The conjugative plasmid, pTA-Mob 2.0, was used as a template to create the reduced versions M1–M4. M3C1 was used as a template to create M5–M8. When available, two clones of the same plasmid were tested (e.g., M1 C1 and C2). Error bars represent the $\pm$ 95% confidence interval (Student's *t*-test was used to carry out pairwise comparisons between pTA-Mob 2.0 (control) and minimized versions of pTA-Mob 2.0: * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$). n = 9 for all strains except n = 27 for pTA-Mob 2.0.
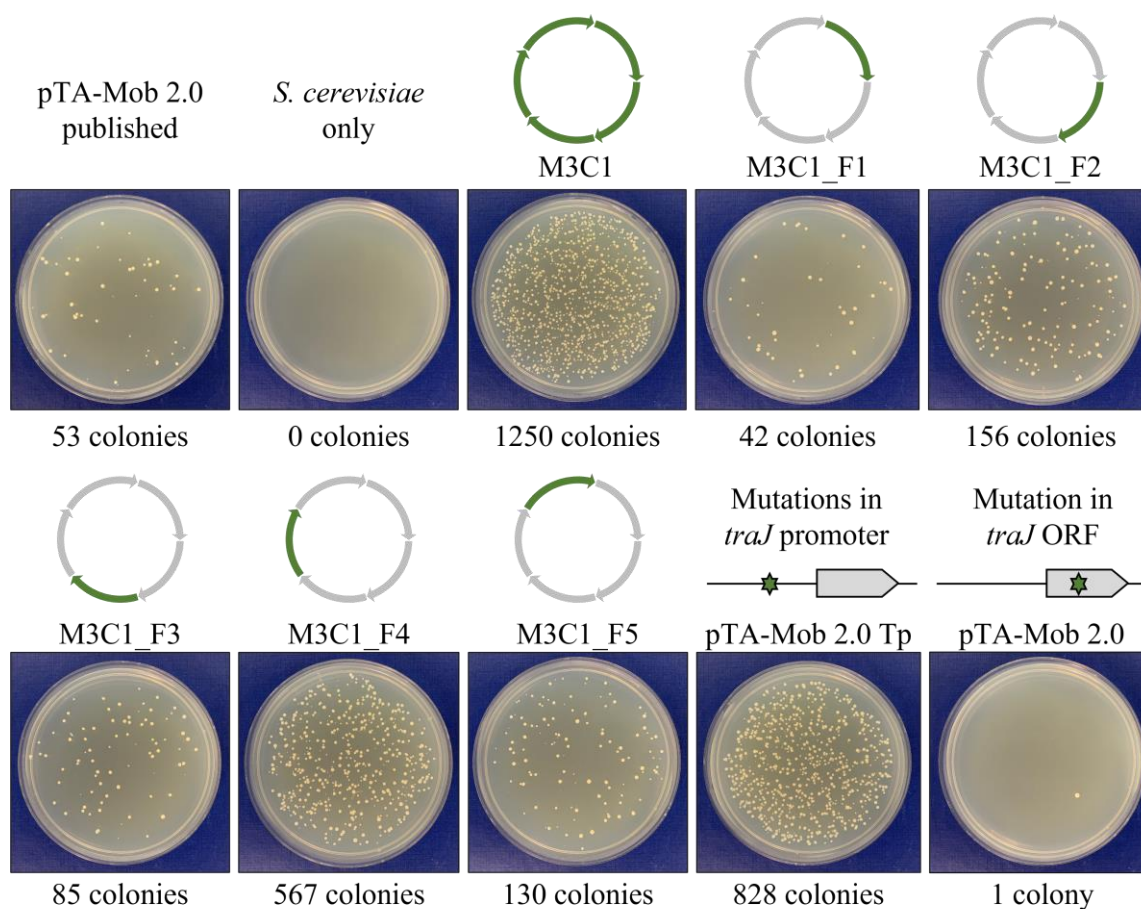
Figure 4-3. Identification of mutations in M3C1 responsible for improved bacterial conjugation to *S. cerevisiae*. *S. cerevisiae* transconjugant colony formation on agar plates containing synthetic complete media lacking histidine following bacterial conjugation of control: pTA-Mob 2.0, M3C1; hybrid: M3C1/M3C2; or mutated pTA-Mob 2.0 Tp/To plasmids from *E. coli*. Schematics above M3C1_F1–F5 display the composition of each hybrid plasmid and which parent plasmid (M3C1/M3C2) fragments were PCR-amplified from. Green arrows represent fragments originating from M3C1, and grey arrows represent fragments from M3C2. In the schematic of *traJ*, green stars indicate mutations in pTA-Mob 2.0 Tp/To plasmids (Tp, a cluster of mutations; and To, a single mutation). Transconjugant colony counts are reported below the plate images.

4.2.2   Creation of the superior conjugative plasmid pSC5

Based on the identified *traJ* promoter mutations, we created the pSC5 plasmid with additional genetic elements to deliver to diverse yeasts and diatoms. The pSC5 plasmid was built based on M3C1 and contained two copies of the nourseothricin resistance gene (y-*nat* and d-*nat*): one optimized for selection in diverse yeast and one for diatoms (Figure 4-4A). Bacterial conjugation efficiency for pSC5 from *E. coli* to *S. cerevisiae* was increased approximately 10- or 23-fold compared to pTA-Mob 2.0 when tested in *cis* (mobilizing itself) or *trans* (mobilizing another plasmid), respectively (Figure 4-4B, Supplemental Table D-5 & D-6). No significant difference in bacterial conjugation efficiency was observed when plasmids were transferred between *E. coli* strains (Figure 4-4B, Supplemental Table D-5 and D-6).

In order to more precisely evaluate the efficiency of bacteria-to-yeast conjugation, we performed additional experiments to monitor the effect of conjugation plasmid-containing *E. coli* on yeast viability. Cells from *E. coli*-to-yeast conjugation experiments were plated on non-selective yeast media supplemented with ampicillin to inhibit *E. coli* growth. More yeast colonies grew when pSC5 was used versus pTA-Mob 2.0 (Supplemental Table D-7), indicating that *E. coli* carrying pSC5 had reduced and/or fewer adverse effects on yeast when they are co-cultured. To determine if the same effect could be observed when different donor cells were used, we performed bacterial conjugation with *Sinorhizobium meliloti* as a donor, as it was previously shown to conjugate to yeast [25]. Similarly, a greater number of yeast colonies grew on non-selective plates when *S. meliloti* harboring pSC5 was used compared to pTA-Mob 2.0 (Supplemental Table D-8). In addition, a greater number of colonies on selective plates were observed following the bacterial conjugation of pSC5 from *S. meliloti* to *S. cerevisiae* compared to pTA-Mob 2.0 (Supplemental Figure D-2, Supplemental Table D-9).

Additional experiments will need to be performed to determine if there is a link between the increased number of yeast colonies on non-selective/selective plates and the
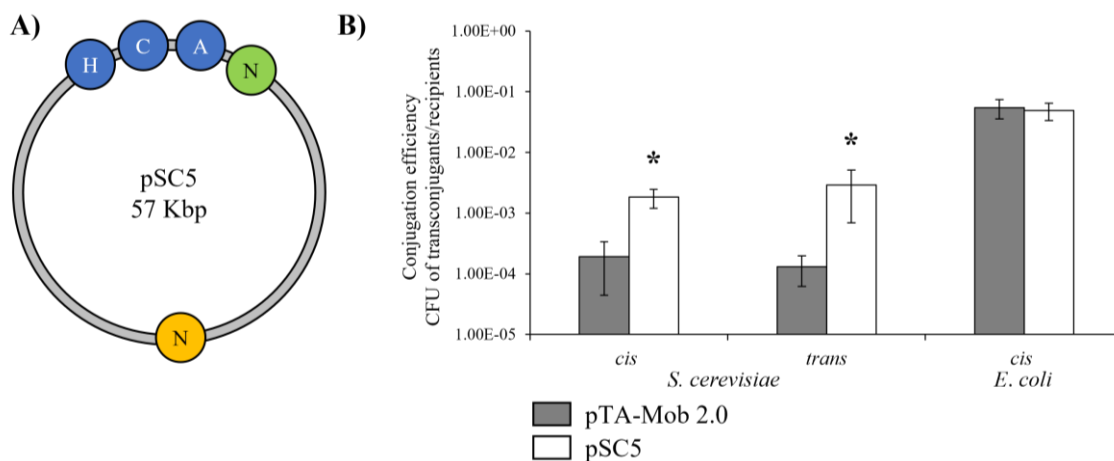
Figure 4-4. Creation and analysis of the pSC5 conjugative plasmid. A) Schematic of pSC5 plasmid map. N – Nourseothricin resistance gene encoded with the standard code for diatom (green) or the alternative yeast nuclear code for *Candida*/*Metschnikowia* (orange; Note – Also correctly translated in *S. cerevisiae*), HCA – *HIS3*, *CEN6*, and *ARSH4* for selection, replication, and maintenance in *S. cerevisiae*. **B)** Bacterial conjugation efficiency of pSC5 (white) compared to pTA-Mob 2.0 (grey) in either a *cis* or *trans* setup from *E. coli* to *S. cerevisiae* (left and middle), or from *E. coli* to *E. coli* in a *cis*-configuration (right). Bacterial conjugation efficiencies are graphed on a $\log_{10}$ scale. Error bars represent the $\pm$ 95% confidence interval (Student's *t*-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). n = 3 and n = 4 for the *cis*- and *trans*-experiment in *S. cerevisiae*, respectively, and, n = 3 for the *cis*-experiment in *E. coli*.

lower expression of *traJ* (Supplemental Figure D-3) in plasmids carrying the promoter mutation.

4.2.3   Bacterial conjugation to diverse yeast species

The significantly improved bacterial conjugation efficiency with the pSC5 plasmid suggests it may be effective for bacterial conjugation beyond a standard laboratory strain of *S. cerevisiae* and may have utility in transferring DNA from bacteria to diverse yeast species. To test the ability of pSC5 to transfer DNA to diverse yeast strains, we selected four *Metschnikowia* and six *Candida* species as conjugative recipients. Previously, we have demonstrated that small DNA fragments (y-*nat* selection marker) can be delivered to most of these species by electroporation [46]. Bacterial conjugation to these diverse yeasts was performed with the same protocol used for *S. cerevisiae*, modified to allow for selection on complete yeast media containing antibiotics. Transconjugant colonies were obtained for all species (Figure 4-5A, Supplemental Figures D-4, and D-5), and 1–8 transformant colonies for each species were genotyped by PCR for the presence of the y-*nat* marker. Of the 10 species tested, seven tested positive by PCR for the presence of the y-*nat* marker, suggesting successful bacterial conjugation had occurred (Supplemental Figure D-6). A plasmid rescue experiment, where total yeast DNA is electroporated into *E. coli*, was performed for selected colonies for each of the seven species, as well as *S. cerevisiae*. pSC5 plasmids from the seven yeast species were successfully recovered in *E. coli*; however, all except those recovered from *S. cerevisiae* showed rearrangements when diagnostic restriction enzyme digestion was performed (Figures 4-5B, C). Furthermore, only the plasmids recovered from *S. cerevisiae* were still able to conjugate (Supplemental Figure D-7).
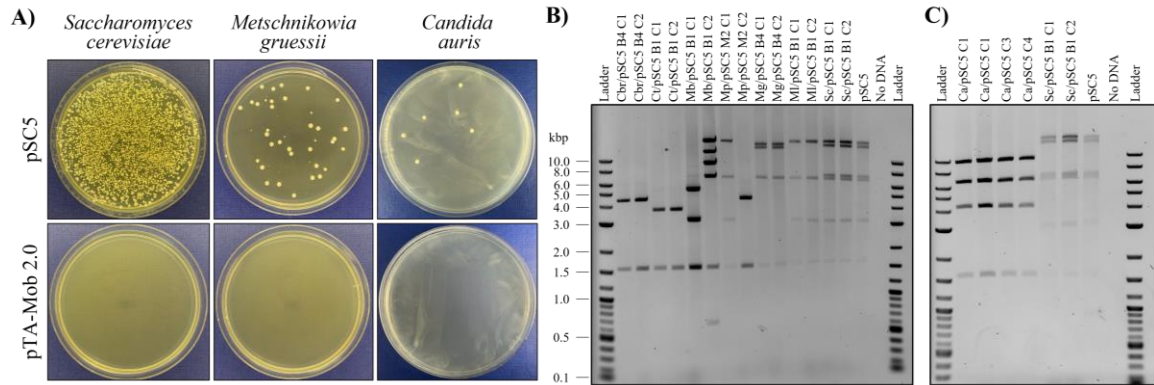
Figure 4-5. Bacterial conjugation of pSC5 to wild yeast strains. A) pSC5 conjugated to *S. cerevisiae* and the wild yeasts *M. gruessi* and *C. auris* on YPD plates supplemented with nourseothricin (100 µg mL⁻¹). **B, C)** Diagnostic double restriction enzyme digestion (EcoRI-HF and AgeI-HF) of pSC5 rescued from yeast strains. The expected band sizes for pSC5 are 20,808-, 15,855-, 7314-, 6848-, 3189-, 1610-, and 6-bp. Cbr – *C. bromeliacearum*, Ct – *C. tolerans*, Mb – *M. borealis*, Mp – *M. pulcherrima*, Mg – *M. gruessi*, Ml – *M. lunata*, Sc – *S. cerevisiae,* and Ca – *C. auris.* Ladder: 2-log ladder.
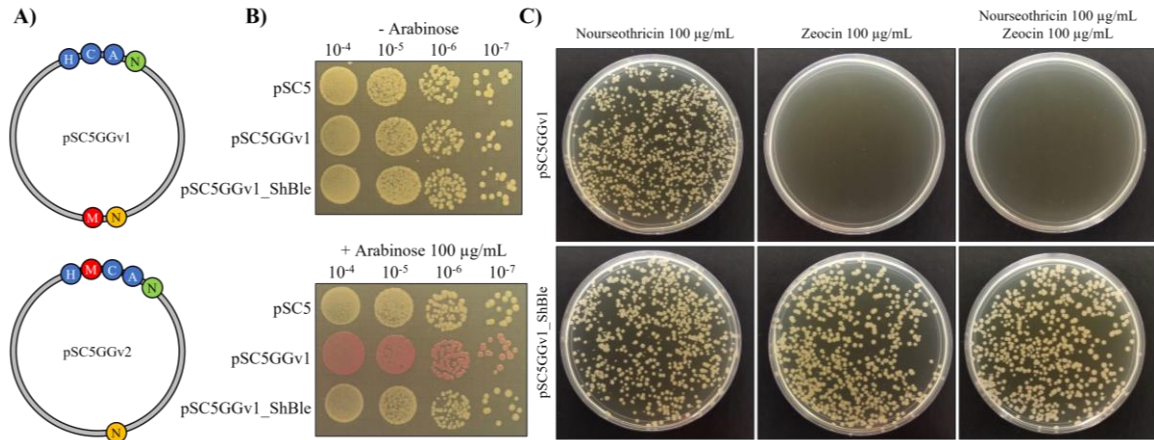
Figure 4-6. Development of a pSC5 plasmid compatible with GG assembly. A) Two versions of the pSC5 plasmid were created. Version 1 (pSC5GGv1) has the *mrfp* landing pad in the middle of the plasmid, and version 2 (pSC5GGv2) has the *mrfp* between the yeast elements *HIS3* and *CEN6-ARSH4*. **B**) Example of GG assembly to insert a gene of interest (*Sh ble*) into pSC5GGv1. pSC5 – original plasmid, pSC5GGv1 – domesticated plasmid, pSC5GGv1_ShBle – a selected *E. coli* colony with *Sh ble* inserted grown on LB plates supplemented with gentamicin (40 µg mL^{-1}). **C**) Bacterial conjugation of pSC5GGv1 and pSC5GGv1_ShBle to *S. cerevisiae*.

4.2.4   Domestication of pSC5 for Golden Gate assembly

Next, we sought to modify the pSC5 plasmid to make it readily amenable for cloning and easily incorporate any desired DNA fragment to facilitate downstream applications of bacterial-to-yeast conjugation. To this end, we eliminated existing BsaI restriction sites from pSC5 and created a single BsaI-based GG cloning-compatible site to enable efficient plasmid manipulation [48,49]. In addition, we incorporated a landing pad with monomeric Red Fluorescent Protein (*mrfp*) driven by an arabinose-inducible promoter (Figure 4-6A). In this modified plasmid, GG assembly can readily be used to replace the *mrfp* gene with any gene of interest, allowing for an easy visual screen for correct gene insertion events (white versus red bacterial colonies).

To validate this system, we inserted a second antibiotic marker (*Sh ble*) for yeast into pSC5GGv1 (Figure 4-6A) to create pSC5GGv1_ShBle, which provides resistance to zeocin. White bacterial colonies were selected (Figure 4-6B) and genotyped with diagnostic MPX PCR and restriction digest (data not shown). These validated colonies were conjugated to *S. cerevisiae* and tested for survival on single or double antibiotic selection (Figure 4-6C). Successful transconjugants which received pSC5GGv1_ShBle were able to grow on media supplemented with zeocin, nourseothricin, or both (Figure 4-6C).

4.2.5   Proof of concept for bacterial conjugation-mediated delivery as an antifungal

To demonstrate that pSC5-based conjugative plasmids could be used as an antifungal, we developed a system where each donor *E. coli* strain carried two plasmids: a control plasmid (pAGE2.0.T), which can be selected on media lacking tryptophan, and either pSC5 or a pSC5-toxic plasmid, which can be selected on media lacking histidine (Figure 4-7A). We used GG assembly to create three pSC5-toxic plasmids, each carrying a gene that should be partially or fully toxic to yeast. To prevent toxicity in *E. coli*, in each toxic gene, we inserted a yeast *ACT1* intron [50]. Next, we cloned the *A. laidlawii* toxic gene [47] into pSC5GGv1 to generate pSC5-toxic1 or a *Haemophilus influenzae* HindII
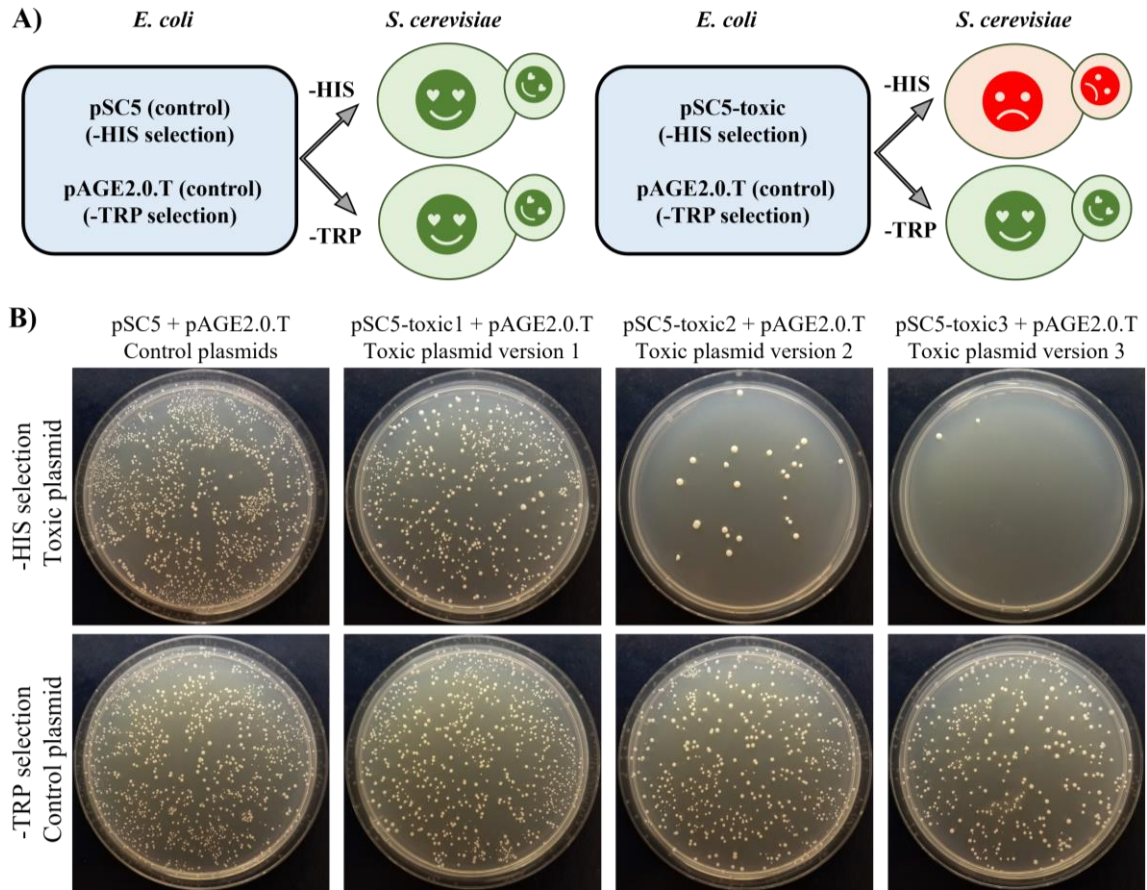
Figure 4-7. Conjugation-based antifungal. A) Diagram illustrating the predicted outcomes on *S. cerevisiae* colony formation following delivery of various plasmids via bacterial conjugation. The control strain (left) harboring pSC5 and pAGE2.0.T selected on synthetic complete media lacking either histidine or tryptophan will survive. Conversely, the experimental strain (right) harboring a pSC5-toxic plasmid and pAGE2.0.T, when selected on a synthetic complete media lacking histidine, will die. **B)** Assay for *S. cerevisiae* colony formation on -HIS or -TRP media following bacterial conjugation with three toxic plasmids. pSC5-toxic1 – partially toxic gene identified in *A. laidlawii* inserted in pSC5GGv1, pSC5-toxic2 – *H. influenzae* HindII restriction gene inserted in pSC5GGv1, pSC5-toxic3 – *H. influenzae* HindII restriction gene inserted in pSC5GGv2.

restriction enzyme gene into pSC5GGv1 and pSC5GGv2 to generate pSC5-toxic2 and pSC5-toxic3, respectively. The pSC5 and pSC5-toxic gene plasmids can act in *cis*, mobilizing themselves, and in *trans*, mobilizing the control plasmid pAGE2.0.T. Using a control *E. coli* strain carrying the plasmids pSC5 and pAGE2.0.T, we observed a similar colony number on both selection plates. For bacterial conjugation using the pSC5-toxic plasmids, substantially fewer colonies grew on media lacking histidine compared to pSC5. The most substantial difference in yeast colony formation was with donor *E. coli* carrying pSC5-toxic3 (Figure 4-7B, Supplemental Table D-10). This provides a proof of concept that bacteria-to-yeast conjugation can be used to deliver plasmid-based antifungals effectively.

4.3   Discussion

Bacterial conjugation-based techniques, such as the one described here, provide a unique and functional method to deliver plasmids between microbial species *in vitro* and *in vivo*. While there are innumerable possible applications for these systems, many have focused on using plasmid-encoded CRISPR-based genetic manipulation systems to modify the genomes of the recipient microbes [19,23,51]. Indeed, CRISPR-based gene targeting and manipulation systems offer a breadth of applications that can be paired with bacterial conjugation (or other methods of DNA delivery, such as phage transduction) to achieve desired manipulation of a target microbial population. The majority of this work to date has focused on bacterial species. For instance, CRISPR-based systems have been used to induce lethal DNA damage in key bacterial pathogens, including *E. coli*, *Staphylococcus aureus*, and *Clostridium difficile*, to effectively eradicate unwanted bacterial populations, including drug-resistant bacteria [13,14,52], and specific pathogenic species or sub-populations [13,16–19,53,54]. In addition to directly killing bacterial populations, CRISPR systems can also be applied to modify virulence determinants to erode microbial pathogenicity [53,55] or alter drug-resistance genes to restore antimicrobial susceptibilities [15,23,56–60]. To enable the application of these CRISPR systems *in vivo*, many have relied on bacterial conjugation or phage transduction to deliver the

relevant CRISPR components [13,17,21–23,61]. While this has been effective for delivery to bacterial strains, it has limited the applications in fungi, which lack well-established tools for bacterial conjugation or virus-based gene delivery [62,63].

To address the bottleneck of improving DNA delivery to yeast, we performed experiments to optimize the conjugative plasmid pTA-Mob 2.0 [28]. We first evaluated whether plasmid derivatives with targeted deletions of the conjugative plasmid could improve DNA transfer to *S. cerevisiae*. After testing 57 single-gene and four cluster-gene deletion plasmids, one with superior conjugative properties (M3C1) was identified. Sequence analysis of M3C1 revealed that in addition to the designed deletions, M3C1 had unintended mutations that were likely introduced during PCR amplification or plasmid assembly. The mutations responsible for improved bacterial conjugation to *S. cerevisiae* were narrowed down to the promoter region of *traJ* (Tp) using a fragment-swapping experiment. Following this discovery, five derivative plasmids of M3C1 were built containing the Tp mutation: four minimized versions (M5–M8) and pSC5 (containing selectable markers for diverse yeast species [46] and diatoms [24]). Each derivative plasmid of M3C1, including the smallest 31 kbp plasmid M8, outperformed the original 57 kbp pTA-Mob 2.0 plasmid when tested for DNA transfer to *S. cerevisiae*. Notably, using pSC5 compared to pTA-Mob 2.0, we observed an increase in bacterial conjugation to *S. cerevisiae* 10- or 23-fold in either a *cis* or *trans* setup, respectively. Yet, no increase in plasmid transfer was observed when pSC5 was conjugated between *E. coli* strains. This improved bacterial conjugation to *S. cerevisiae* could be partially explained by the increased *S. cerevisiae* viability during the co-culture bacterial conjugation step when plasmids harboring the Tp mutation are used. The same effect was observed when *S. meliloti* was used as a conjugative donor, suggesting the mechanism may be independent of the bacterial host. We also demonstrated that the Tp mutation results in a lower expression of the *traJ* gene. TraJ has been demonstrated as an essential conjugative protein that negatively autoregulates the expression of the relaxase operon [64]. Therefore, decreased expression of *traJ* could significantly affect the expression of all the conjugative machinery proteins. Further investigation will focus on resolving the link

between *traJ* downregulation and increased yeast viability or DNA transfer during the co-culture bacterial conjugation step.

The significantly improved pSC5 plasmid allowed DNA transfer to seven *Metschnikowia* and *Candida* yeast species, though relatively few colonies were obtained for each. One explanation for the low conjugative transfer could be that the *S. cerevisiae* centromere and the origin of replication were not functional in these yeasts. In such a case, the survival of these yeasts would only be possible if the conjugative plasmid was integrated into the yeast genome. Using a plasmid rescue experiment, we showed that plasmids could be recovered in *E. coli*, although none had the correct size or ability to conjugate. Since *E. coli* can assemble linear fragments into plasmids [65], it is most likely that some of the linear yeast fragments with integrated conjugative plasmids were assembled into plasmids in *E. coli*. Despite being unable to replicate as an episome in diverse yeasts, the improved conjugative plasmids, especially pSC5GGv1_ShBle with two antibiotic-resistance genes, provide a great initial resource for DNA delivery. For applications where replicative plasmids are necessary for the yeast species of interest, species-specific origins of replication and centromeres must be identified and incorporated into the conjugative plasmid, as was done for *S. cerevisiae* [66,67].

Our improved conjugative plasmids hold promise as a novel antifungal. As a proof of concept, we cloned restriction nucleases onto the pSC5GGv2 plasmid and demonstrated that over 99% of yeast cells that receive the plasmid could be eliminated. However, additional improvements in the bacterial conjugation efficiency will need to be achieved before this technology can be used in antifungal treatments. In the future, our GG-compatible plasmids can be engineered with programmable systems such as CRISPR/Cas9 to target specific yeast strains. This coincides with the development and optimization of numerous CRISPR-based editing platforms optimized for diverse yeast species [68–72], including *Candida* pathogens [73]. Recent work has demonstrated the utility of CRISPR systems for modifying fungal genes involved in virulence [74–81] and antifungal drug resistance [74,77,82,83] in diverse *Candida* pathogens, and combining these CRISPR systems with this trans-kingdom bacterial conjugation system could facilitate the delivery of CRISPR to fungi in different environmental contexts.

4.4　Material and Methods

4.4.1　Experimental design

Experimental design is shown in Figure 4-1.

4.4.2　Microbial strains and growth conditions

Saccharomyces cerevisiae VL6−48 (ATCC MYA-3666: *MATα*, *his3-Δ200*, *trp1-Δ1*, *ura3−52*, *lys2*, *ade2−101*, *met14*, psi$^+$, cir^0) was grown in yeast media supplemented with ampicillin (100 μg mL^{-1}) as previously described in Section 2.4.1; or grown with selection on either: 1) yeast synthetic complete media lacking histidine supplemented with adenine hemi-sulfate, 2) yeast synthetic complete media lacking tryptophan, 3) 2 x YPDA supplemented with nourseothricin (100 μg mL^{-1}), or 4) 2 x YPDA supplemented with zeocin (100 μg mL^{-1}). Solid yeast media contained 2% agar. All yeast spheroplast preparation and transformations were performed as previously described in Section 2.4.5. *Escherichia coli* (Epi300, Lucigen) was grown as previously described in Section 2.4.1 supplemented with appropriate antibiotics (gentamicin (40 μg mL^{-1}) and chloramphenicol (15 μg mL^{-1})). Solid media contained 1.5% agar. For the transformation of *E. coli*, SOC media was used during the recovery time. Diverse yeasts, *Metschnikowia gruessii* (H53), *Metschnikowia pulcherrima* (CBS 5833), *Metschnikowia lunata* (BS 5946), *Metschnikowia borealis* (SUB 99-207.1), *C. auris*, *Candida tolerans* (UWOPS 98-117.5), *Candida bromeliacearum* (UNESP 00-103), *Candida pseudointermedia* (UWOPS 11-105.1), *Candida ubatubensis* (UNESP 01-247R), and *Candida* aff. *bentonensis* (UWOPS 00-168.1) were grown at 30 °C in 2 x YPDA (all diverse yeasts were obtained from Dr. Marc-Andre Lachance collection at Western University except *C. auris* came from https://wwwn.cdc.gov/arisolatebank/; accession number: SAMN05379609). *Sinorhizobium meliloti* (Rm4123 R$^-$; obtained from Dr. Finan Lab, McMaster University) was grown at 30 °C in LBmc media (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl, 0.301 g L^{-1} MgSO$_4$, and 0.277 g L^{-1} anhydrous CaCl$_2$) supplemented with appropriate

antibiotics (gentamicin 40 μg mL^{-1} and streptomycin 100 μg mL^{-1}). Solid media
contained 1.5% agar.

4.4.3   Plasmid construction

4.4.3.1   PCR amplification

Plasmid fragments were amplified with GXL polymerase according to the manufacturer's
instructions using annealing temperatures between 50 and 60 °C and 25–30 cycles.

4.4.3.2   Plasmid assembly in yeast

Plasmids were assembled in yeast, as previously described [28]. Primers for deletion
plasmids are listed in Supplemental Table D-1, and all primers and templates used to
generate other plasmids are listed in Supplemental Table D-2: **(1) Single-gene/fragment
deletion plasmids:** pTA-Mob 2.0 plasmid was used as a template. Each plasmid was
created with nine standard fragments, as previously described [28], and two additional
fragments designed as shown in Supplemental Figure D-1. **(2) Minimized plasmids
(M1–8):** Eight minimized conjugative plasmids (M1–8) were designed based on the
results obtained for the pTA-Mob 2.0 deletion plasmids. pTA-Mob 2.0 or M3C1 plasmid
was used as a template for PCR fragments listed in Supplemental Table D-2. **(3)
M3C1_F1–F5 hybrid plasmids:** These plasmids were assembled by swapping the
fragments between M3C1 and M3C2. **(4) pTA-Mob 2.0 Tp and pTA-Mob 2.0 To:**
Primer-mediated mutagenesis was performed to introduce each mutation (Tp- in *traJ*
promoter and To- in *traJ* ORF) into pTA-Mob 2.0. **(5) Superior conjugative plasmid
(pSC5):** The pSC5 plasmid was derived from the M3C1 plasmid by the addition of two
versions of the nourseothricin N-acetyltransferase (*nat*) gene, which provides resistance
for the antibiotic nourseothricin. The first version was amplified from a plasmid pTA-
Mob-NAT (unpublished, Karas lab), allowing selection in diatoms and referred to as d-
nat; and the version was amplified from pGMO1 (unpublished, Karas lab), which

contained an alternative genetic code for selection in diverse yeasts as previously described [46] and referred to as y-*nat*. The remaining fragments of the pSC5 plasmid were amplified from M3C1, as previously described [28]. **(6) pTA-Mob 2.1/pSC5.1:** These two plasmids were created lacking the second copy of *traJ* located in the vector backbone. **(7) Domesticated pSC5 (pSC5GGv1/v2):** Two BsaI cut sites within the *fcpD* promoter and *traC1* ORF were removed from pSC5 using primer-mediated mutagenesis. A landing pad, consisting of an *mrfp* gene driven by an arabinose-inducible pBAD promoter and a terminator, was amplified from pAGE2.0-I using primers designed with new BsaI cut sites and homology either to directly downstream the native I-SceI restriction site (pSC5GGv1) or within the *HIS3*/*CEN6*/*ARSH4* element of the vector backbone (pSC5GGv2).

4.4.3.3 Golden Gate assembly

(1) GG assembly. For GG assembly, 20 fmol of plasmid and insert were mixed in a 15 μL reaction with 1.0 μL T4 DNA ligase and 0.5 μL BsaI-HF V2, using the following conditions: 10 cycles of 37 °C for 5 min and 16 °C for 10 min followed by incubation at 37 °C for 5 min, 80 °C for 10 min, and infinite hold at 12 °C. Primers are listed in Supplemental Table D-2. (2) pSC5GGv1_ShBle. The zeocin resistance marker (*Sh ble*) cassette was amplified with flanking BsaI cut sites from pRS32 (unpublished, Shapiro Lab), and GG assembly was performed with pSC5GGv1. The primers used are listed in Supplemental Table D-2. (3) pSC5-toxic1, pSC5-toxic2, and pSC5-toxic3. Three versions of toxic plasmids were created to kill yeast cells, one with an *Acholeplasma laidlawii* toxic gene (ACL0117) [47] and two with the restriction enzyme HindII. The *A. laidlawii* and HindII cassettes contained an *ACT1* yeast intron, flanking BsaI cut sites, and either an *A. laidlawii* or HindII toxic gene. The *A. laidlawii* toxic gene cassette was amplified in three fragments: the *ACT1* yeast intron from *S. cerevisiae* VL6−48 and the toxic gene in two halves from *A. laidlawii* PG-8A with primers listed in Supplemental Table D-2. The *A. laidlawii* toxic gene cassette was constructed through a hierarchical GG assembly. First, the *ACT1* yeast intron and the second half of the *A. laidlawii* toxic

gene were assembled by GG assembly, and then 1 μL of the product was used as a template for PCR amplification of the joined fragments. Next, GG assembly was performed with 20 fmol of the PCR product with the first half of the toxic gene, and the complete toxic gene cassette was PCR-amplified. The fully constructed cassette was then mixed with pSC5GGv1, and GG assembly was performed. The HindII cassette split by the *ACT1* intron was flanked by the *URA3* promoter and terminator and was synthesized (BioMatik, Canada), then PCR-amplified and used in GG assembly with pSC5GGv1 or pSC5GGv2.

4.4.4   Plasmid analysis

Screening in yeast. Following yeast assembly of the pTA-Mob 2.0 deletion plasmids, 20 individual yeast colonies were passed twice on agar plates containing synthetic complete media lacking histidine, and DNA was isolated and screened by MPX PCR using the Qiagen Multiplex Kit according to the Qiagen Multiplex PCR Handbook. For all other plasmids following yeast assembly, colonies were pooled rather than individually screened. **Transformation to *E. coli*.** Total DNA was isolated as previously described in Section 2.4.2.2. Isolated DNA (0.5–2 μL) was added to 40 μL of *E. coli* electro-competent cells and electroporated using the Gene Pulser Xcell Electroporation System (2.5 kV voltage, 25 μF capacitance, and 200 Ω resistance). Following a recovery in 1 mL of SOC media for 1 h at 37 °C (225 RPM), a 100–250 μL aliquot of the transformants was plated on LB media supplemented with gentamicin (40 μg mL^{-1}). The cells transformed with GG-compatible plasmids (pSC5GGv1/v2, pSC5-toxic1, pSC5-toxic2, and pSC5-toxic3) were instead plated on LB plates containing gentamicin (40 μg mL^{-1}) and arabinose (100 μg mL^{-1}). **Screening in *E. coli*.** For GG-compatible plasmids, white colonies were screened for insertion of the cassette of interest by MPX PCR. For all other assembled plasmids, the transformed *E. coli* was pooled and conjugated to *S. cerevisiae*, the DNA was isolated, and transformed back to *E. coli* to focus screening on functional conjugative plasmids. Once in *E. coli*, all plasmids were genotypically screened using MPX PCR and restriction enzyme digest analysis. **Sequencing.** The plasmids, pTA-Mob

2.0 Tp/To, underwent Sanger DNA sequencing (London Regional Genomics Centre at Robarts Research Institute) to ensure the introduction of the correct mutations using the primers listed in Supplemental Table D-2. Selected plasmids were sequenced at CCIB DNA Core at Massachusetts General Hospital or the Western University sequencing facility.

4.4.5   Bacterial conjugation

Both donor (*E. coli*) and recipient (*S. cerevisiae*) strains were prepared and frozen prior to bacterial conjugation experiments. For *E. coli* strains, saturated overnight cultures inoculated with a single colony were diluted to OD_{600} of 0.1 in 50 mL of LB media supplemented with appropriate antibiotics (Table 4-1) and grown until an OD_{600} of 1.0 was reached. The cells were pelleted (3000 x RCF, 15 min) in a 50 mL Falcon tube and resuspended in 500 μL ice-cold 10% glycerol. Then, 100 μL aliquots in Eppendorf tubes were frozen in a -80 °C ethanol bath and stored at -80 °C. For *S. cerevisiae* recipient strain preparation, a culture was started from a single colony and grown in 5 mL of 2 x YPDA media supplemented with ampicillin (100 μg mL^{-1}) for 7 h. After, this culture was diluted in 50 mL of 2 x YPDA media supplemented with ampicillin (100 μg mL^{-1}) and grown until an OD_{600} of 3.0 was reached (~17 h). The cells were pelleted (3000 x RCF, 5 min) in a 50 mL Falcon tube and resuspended in 1 mL of ice-cold 10% glycerol. Then, 250 μL aliquots in Eppendorf tubes were frozen in a -80 °C ethanol bath and stored at -80 °C.

On the day of bacterial conjugation, conjugation plates (20 mL, 1.8% agar, 10% LB media, synthetic complete yeast media lacking histidine) were dried for 30 min. Aliquots of the donor (*E. coli*) and recipient (*S. cerevisiae*) strains were removed from the freezer and thawed on ice for approximately 20 min. Next, 50 μL of *S. cerevisiae* was added to the 100 μL of *E. coli* and mixed by gentle pipetting before being transferred to the plate and spread evenly. Alternatively, when the yeast toxic plasmids were being tested, 10 μL of the recipient *S. cerevisiae* strain was used. Once dried, the plates were incubated at 30 °C for 3 h, or 12 h when wild yeast strains were used as the recipient. The

plates were scraped with 2 mL of sterile double-distilled water (sddH$_2$O), mixed by vortexing for 5 s, and 100 μL plated on respective selection media (25 mL, 2% agar supplemented with ampicillin 100 μg mL^{-1}) listed in Table 4-1. In the case of wild yeast strains, they were plated on 1 x YPDA media supplemented with nourseothricin (100 μg mL^{-1}), and two technical replicates of each dilution (10^0–10^{-1}) were plated on selective plates. For experiments evaluating bacterial conjugation of pSC5 in *cis* and *trans*, dilution series of 10^0–10^{-2} were generated and plated on selective media while dilution series of 10^{-4}–10^{-7} were generated and plated on non-selective media (1 x YPDA supplemented with ampicillin 100 μg mL^{-1}); and two technical replicates were plated for each dilution.

4.4.6　RNA isolation and quantitative reverse transcriptase-polymerase chain reaction

For RNA isolation, each *E. coli* strain carrying either conjugative plasmid, pTA-Mob 2.1 or pSC5.1 were grown in LB media supplemented with gentamicin (40 μg mL^{-1}) overnight at 37 °C shaking at 225 RPM. In the morning, RNA was isolated as previously described in Section 3.5.8. Following DNase treatment with TURBO DNA-free Kit (Invitrogen), the RNA concentration and integrity were verified [43].

cDNA was prepared from 500 ng of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using six biological and three technical replicates on a ViiA7 system of QuantStudio Real-Time PCR System using the SYBR™ Select Master Mix under the following conditions: 50 °C for 2 mins, 95 °C for 2 mins followed by 40 cycles of: 95 °C for 1 s, 60 °C for 30 s. Expression levels were normalized against two reference genes (*rrsA* and *cysG*), as previously described [29]. Primer sequences used for the qRT-PCR expression analyses are listed in Supplemental Table D-2.

4.4.7   Statistical analysis

The pairwise comparisons between groups were made using Student's *t*-test with either equal or unequal variance based on the result of an F-test. Data were expressed as either a $\pm 95\%$ confidence interval, mean $\pm$ standard error of the mean, or mean $\pm$ standard deviation of at least three biological replicates. The tests were considered statistically significant when $p < 0.05$ ($*$), $p < 0.01$ ($**$), or $p < 0.001$ ($***$).

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Chapter 5

5   General Discussion

The development of WGE for mitochondria could enable the study of the origins of eukaryotes, progress treatments for mitochondrial diseases in humans, and produce superlative eukaryotes for industry. However, bottlenecks to a synthetic biology approach for whole mitochondrial genome engineering exist, including the need for standardized genetic engineering methods and inept DNA delivery efficiencies to eukaryotes. This thesis begins to address these limitations by presenting protocols for rapidly engineering diatom mitochondrial genomes and genetic tools for improved DNA delivery to eukaryotes. The whole mitochondrial genome engineering method enables the inexpensive and rapid generation of derivative mitochondrial genomes. The superior conjugative plasmid, pSC5, improves bacterial conjugation to eukaryotes and provides an amenable system for engineering efforts, such as redirecting DNA delivery from the nucleus to the mitochondria. Additionally, its increased bacterial conjugation efficiency offers an initial tool for DNA delivery to diverse yeasts relevant to medicine and industry. The technologies presented represent the initial steps toward whole mitochondrial genome engineering that have important implications in future basic scientific research, medicines, and biotechnologies.

5.1   Design-build-test: Mitochondrial genome cloning and engineering

Efficient mitochondrial genome engineering efforts require cloned genomes for template DNA to modify and standardized protocols to create variant mitochondrial genomes reproducibly. The mitochondrial genomes of two diatoms, *P. tricornutum* and *T. pseudonana*, were cloned in *S. cerevisiae* and *E. coli* using PCR-based and TAR cloning methods. The cloning was efficient for both species despite differences in the characteristics of the mitochondrial DNA, such as size, type of repetitive elements, and G+C% content. Although both cloning methods were successful, each has advantages

and limitations. The TAR cloning method directly captures an endogenous mitochondrial genome but is labor-intensive, time-consuming, and inefficient relative to the PCR-based method. However, this method produced a *bona fide* mitochondrial template for optimizing a rapid and efficient PCR-based engineering protocol. The PCR-based engineering protocol can be applied to any species with sequencing data and is currently cheaper than DNA synthesis. It is adaptable and can produce many genetic modifications, including single nucleotide polymorphisms, insertions, deletions, addition and removal of selection markers, and introduction of entire biosynthetic pathways. For instance, the first designed modification of *P. tricornutum* and *T. pseudonana*'s mitochondrial genomes included the removal of repetitive DNA regions that further simplified PCR amplification for future iterations of genome engineering. However, PCR-based cloning and engineering methods can inadvertently introduce mutations or be incapable of amplifying large repetitive sequences.

The PCR-based engineering protocol requires approximately ten days; however, each cycle introduces ~1–2 mutations per 20 kbp of sequence (Tables 2-2 and 3-2). This mutation rate is significant because mitochondrial genomes are generally small and compact, which increases the likelihood that a mutation could impact endogenous genetic structures vital to its function. Prior to delivery experiments, modified genomes should be sequenced to identify any mutations, which can then be individually corrected or screened out. In the future, the PCR-based engineering method could be replaced with a GG assembly method [1] to increase the genetic tractability of derivative mitochondrial genomes while maintaining the flexibility of PCR-based modifications.

5.2   Mitochondrial plasmid stability and host burden

Faithful maintenance of cloned mitochondrial genomes (i.e., plasmids) in host organisms is crucial for downstream experimentation. After successfully cloning two diatom mitochondrial genomes in *S. cerevisiae* and *E. coli*, their plasmid stability and host burden were examined. Often shared genetic elements, aberrant gene expression, or repetitive sequences can cause cellular dysregulation, host burden, and plasmid instability

[2–9]. Mitochondrial plasmids posed no observable host burden in either host species despite RNA sequencing in *E. coli* detecting gene expression from both diatom mitochondrial genomes (Supplemental Tables C-3 and C-4). Host burden was observed via reduced end-point culture density of all plasmids maintained at a high copy number in *E. coli* (Figures 3-2B and 3-3G, H). Although maintaining problematic plasmids at a high copy number is generally poor practice, it is excellent at amplifying low-level host burden or plasmid instability to identify troublesome regions.

Despite minimal host burden, *T. pseudonona*'s mitochondrial plasmid had detectable instability, likely due to its repetitive elements. Both diatom mitochondrial genomes contain segregated repetitive elements but of different types. Specifically, *P. tricornutum* contains 35.5 kbp of direct repeats, whereas *T. pseudonana* contains 4.8 kbp of inverted repeats [10]. The mutated *T. pseudonana* mitochondrial plasmids were sequenced, and large deletions were observed in proximity to the inverted repeat regions implicating their involvement. Repetitive DNA sequences in mitochondrial genomes are essential for interactions with nucleoproteins, proper genome segregation during cellular division, and often contain replication origins [11]. However, plasmids containing inverted repeats within bacterial hosts are troublesome and unstable [8,9] because they can produce secondary structures and cause replication fork stalling, increasing the likelihood of recombination events [5–8]. Alternatively, these problematic plasmids can be stably maintained in eukaryotic hosts [12–14] and transferred to bacteria temporarily for high-yield DNA isolation or mitochondrial DNA delivery experiments. Notably, the mutation frequency of T. *pseudonana*'s cloned mitochondrial genome is sufficiently low that it would not hinder potential delivery experiments provided DNA is screened following propagation in *E. coli*.

5.3   Improving bacterial conjugation

Bacterial conjugation delivers DNA to diverse cell types but with highly variable efficiencies between species [15–18]. For example, bacterial conjugation between Gram-negative bacteria is highly efficient and delivers DNA to 10–30% of possible recipient

cells, whereas bacterial conjugation to eukaryotes, such as *S. cerevisiae*, ranges from 10^{-5}–10^{-7} transconjugants per recipient cell [15,16,19]. While conjugative plasmids can deliver DNA to diverse cell types, if transferred to a eukaryotic host in nature, they would only persist transiently because they lack the eukaryotic genetic elements required for their maintenance, replication, and selection [15]. Therefore, our group and others have adapted conjugative plasmids for various cell types by adding eukaryotic genetic elements for algal [16,18], fungal [15–17,19,20], plant [15], and mammalian cells [15,21,22]. However, bacterial conjugation efficiencies to eukaryotic hosts using these engineered conjugative plasmids remain drastically lower relative to their bacterial counterparts. In eukaryotes, continued propagation of each plasmid can only occur vertically, inherited by direct genetic descendants of recipient cells. Whereas, in bacterial hosts, following plasmid transfer to a recipient cell, that cell can then become a donor and continue the propagation of the plasmid horizontally to other potential recipient cells. Without a selection pressure to acquire, maintain and transfer conjugative plasmids, these systems cannot naturally evolve for efficient transfer to eukaryotes, and therefore, many conjugative systems could likely be optimized using a synthetic biology approach to massively increase the efficiency of DNA delivery to various species.

Multiple factors have been examined to improve bacterial conjugation efficiency to yeast, such as optimizing protocol parameters [19], engineering the genetic background of donor cells [23], and assaying modifications made to conjugative plasmids [15]. For instance, bacterial conjugation efficiency can differ by orders of magnitude by adjusting the donor-to-recipient cell ratio [19]. Others have assayed the Keio *E. coli* knockout library and identified donor chromosomal gene deficiencies that improve bacterial conjugation to *S. cerevisiae* [23]. Future research could investigate potential yeast gene deficiencies that could make *S. cerevisiae* an improved recipient by screening the Yeast Knockout (YKO) Collection. Here, a deletion plasmid library of pTA-Mob 2.0 was created to improve DNA delivery to yeast and begin generating the resources necessary for adapting and optimizing bacterial conjugation to different cell types and subcellular localizations. The bacterial conjugation efficiency to yeast of each deletion plasmid in the library was assessed, and the data generated was used to create various

streamlined conjugative plasmids by combining multiple non-essential deletions. This series of conjugative plasmids drastically reduced in size led to the identification of the highly transmissible conjugative plasmid, pSC5.

5.3.1 Improved bacterial conjugation efficiency to eukaryotes

Efficiency of DNA delivery is one of the most significant limitations of any genetic engineering effort in synthetic biology. The engineered plasmid pSC5 improves bacterial conjugation ~23-fold to *S. cerevisiae* with no significant effect on conjugation between bacteria. Improved bacterial conjugation to yeast was linked to a cluster mutation in the *traJ* promoter region of M3C1, which was identified using a plasmid fragment-swapping experiment, that was subsequently incorporated into pSC5 (Figure 4-3). TraJ is an essential conjugative protein with multiple functions. It is a negative autoregulatory transcription factor that partly controls the expression of the relaxase operon [24], is a member of the relaxosome protein complex that binds the *oriT*, and orients the relaxase protein, TraI, to the plasmid nick site [15,25–28]. qRT-PCR revealed reduced *traJ* mRNA in *E. coli* harboring pSC5.1 compared to pTA-MOB 2.1, but what effect this has on all the mobilization proteins under its regulation is yet to be determined.

Increased transconjugant yield was also found using an alternative bacterial host, *S. meliloti*, suggesting that improved bacterial conjugation to yeast was donor independent. Furthermore, regardless of donor bacterial species, some proportion of the increased bacterial conjugation efficiency in pSC5 resulted from reduced co-culture toxicity to the recipient, *S. cerevisiae*, when the *traJ* promoter region was mutated. Future research should seek to minimize the host burden and toxicities associated with bacterial conjugation on both host and recipient cells to maximize the efficiency of DNA delivery. This could be achieved partially by streamlining conjugative plasmids (Table 4-1; Figure 4-2) and creating inducible conjugative systems to reduce and temporally control the metabolic costs and toxicities of bacterial conjugation.

Many eukaryotic organisms with industrial potential, including unconventional yeasts, are overlooked because they lack genetic tools or DNA delivery methods. Unconventional yeasts are of interest to industry for their unique contributions to the fermentation process, bioremediation qualities, and production of industrial ethanol, foodstuffs, and probiotics [29–31]. Other species can act as opportunistic pathogenic yeasts (e.g., *Candida* – candidiasis), which are a growing concern in medicine [32–35]. *Candida auris* is an emerging *Candida* pathogen attracting attention because of its resistance to multiple conventional antifungal drugs [34,35]. pSC5 was shown to deliver DNA to unconventional yeast species that were previously unreported to have undergone bacterial conjugation. We repurposed pSC5 as a novel antifungal by loading the plasmid with different genes toxic to yeasts [36,37] and demonstrated that 99% of yeast that received the plasmid during bacterial conjugation were no longer viable. While for *S. cerevisiae*, bacterial conjugation efficiencies of 1 x 10^{-3}–10^{-4} transconjugants per recipient cell are bordering satisfactory for developing a novel therapeutic (Figure 4-4). The need for continued improvement is highlighted by drastically lower bacterial conjugation efficiencies for unconventional yeasts, such as *Metschnikowia*, range between 1 x 10^{-6}–10^{-7} transconjugants per recipient cell, which at its current state would have a negligible impact (Supplemental Figure D-5). Despite still being too early for an efficacious antifungal treatment, pSC5 is still an excellent tool for initial DNA delivery efforts in unconventional yeasts and as a starting point for engineering efforts to attenuate DNA delivery to the mitochondria.

5.3.2 Modulating bacterial conjugation specificity for mitochondrial DNA-delivery

Two approaches have been proposed for mitochondrial transformation using bacterial conjugation: 1) intra-cellular bacterial conjugation and 2) engineering conjugative proteins with mitochondrial localization signals [38,39]. In either scenario, bacterial conjugation is performed under suboptimal conditions. For instance, the non-standard conditions of cytosolic bacterial conjugation and the engineering of mobilization proteins likely reduce the efficiency of DNA transfer. pSC5 is a strong candidate conjugative

plasmid for either approach because its improved DNA delivery to yeast could mitigate the anticipated reduction to bacterial conjugation efficiencies. Additionally, it is a highly malleable and engineerable system, which we have demonstrated in our lab by using pSC5's parent plasmid pTA-Mob 2.0 to generate streamlined plasmids (Figure 4-2) and plasmid libraries (Supplemental Tables D-1, and D-3), complement gene knockouts in *trans*, and perform bacterial conjugation with an engineered relaxase harboring an N-terminal nuclear localization signal (unpublished, Karas lab). In any case, modulation of bacterial conjugation for mitochondrial transformation will require a highly efficient starter plasmid because of the trade-off between bacterial conjugation efficiency and specificity.

Previously it has been shown that *E. coli* can be engineered to evade vacuolar degradation [40] and successfully conjugate in the cytosol of a eukaryotic cell to another bacteria [41]. It has also been demonstrated that bacterial conjugation can successfully transform isolated mitochondria [42]. These two findings suggest that cell fusion and bacterial conjugation could be combined to generate a novel method of DNA delivery to the mitochondria. Intracellular mitochondrial DNA delivery by bacterial conjugation is an attractive alternative method because bacterial conjugation has evolved for efficient delivery between bacteria, not eukaryotes. This bias may enhance the specificity of DNA delivery to the "bacteria-like" mitochondria rather than the nuclear membrane. Furthermore, the pilus could directly interact with the mitochondrial membranes during cytosolic bacterial conjugation bypassing the mitochondrial transport machinery entirely. It was found that mutations in the *traJ* promoter region increased yeast recipient cell viability (Supplemental Tables D-7 and D-8). Future research into this approach for mitochondrial transformation may benefit from low-toxicity donor strains for intracellular bacterial conjugation, such as those harboring pSC5.

The other bacterial conjugation-based approach for mitochondrial DNA delivery is the engineering of key mobilization protein machinery with the addition of mitochondrial localization signals. Multiple conjugative proteins, which have been reported to interact and co-translocate with pDNA to the recipient cytoplasm, could be engineered with mitochondrial localization signals and tested. These include primase

(TraC), relaxase (TraI), recombinase (RecA), and single-stranded binding proteins (SSBs) [15,43–45]. However, engineering any conjugative mobilization protein will likely diminish its function [15]. For example, relaxase is a complex protein responsible for 1) recognizing and nicking the *oriT*, 2) covalently binding the plasmid DNA, 3) co-translocating with DNA from donor to recipient cell, 4) scanning incoming single-stranded DNA, 5) nicking the incoming strand, and 6) re-circularizing the single-stranded DNA after transfer [15,46–48]. The hope is that engineering mobilization proteins with a mitochondrial localization signal will redirect DNA delivery from the nucleus to the mitochondria. However, superior conjugative systems will be crucial to offset the decreased efficacy of DNA transfer caused by modifying any key mobilization proteins with mitochondrial localization signals.

5.4   Whole mitochondrial genome engineering

Whole mitochondrial genome engineering is the cumulation of standardized genome engineering protocols and mitochondrial transformation methods. While the number of mitochondrial genomes cloned and protocols for engineering them has steadily increased, novel methods of DNA delivery have been stagnant [49]. Mitochondrial transformation of single-celled eukaryotes has been exclusively performed by biolistic bombardment (i.e., gene gun delivery), a destructive method that mechanically punctures cell membranes, leaving the vast majority of cells unviable. Only *S. cerevisiae* [50–54], *C. reinhardtii* [54–58], and *C. glabrata* [59] have successfully undergone mitochondrial transformation procedures. Each species transformed has a linear mitochondrial genome, recoverable respiratory deficient mutants, and cell walls reflecting the traits and conditions enabling successful transformation using biolistic-mediated DNA delivery [51,54,60]. Alternative methods, such as electroporation, have been attempted, which are effective on isolated mitochondria but render the mitochondria permanently inactivated in whole-cell experiments [38,42,61,62]. Highly efficient, generalizable, and less destructive DNA delivery methods are needed to transform the mitochondria of diverse eukaryotes and to enable whole mitochondrial genome delivery. Bacterial conjugation is

a promising alternative method that occurs entirely *in vivo*, is highly engineerable, and is indiscriminate in DNA delivery that could be used for diverse cell types and subcellular localizations.

5.5   Conclusion

The future of synthetic biology is complete genetic control of cellular systems, which has been demonstrated in prokaryotes and begun for eukaryotes. However, in eukaryotes, WGE is limited to the nucleus and requires developing the same tools for the mitochondria. This thesis presents methods to rapidly engineer mitochondrial genomes and an improved conjugative plasmid for DNA delivery to eukaryotes. The mitochondrial genome engineering platform presented in Chapters 2 and 3 offers an alternative method to DNA synthesis for deriving designer mitochondrial genomes. The method is inexpensive and rapid, requiring 10-14 days. It is efficient, requires minimal screening, and is adaptable for introducing diverse mutations. The mitochondrial genomes cloned as plasmids were generally stable and non-toxic despite detectable low-level mRNA expression in *E. coli*. These findings support the feasibility of engineering these diatom's mitochondrial genomes comprising the design-build phases of the DBT cycle. Nevertheless, it still requires the development of efficient methods of mitochondrial transformation in these diatom species.

The study presented in Chapter 4 improved the efficiency of DNA delivery by bacterial conjugation to eukaryotes. The deletion plasmid library of pTA-Mob 2.0 characterized the essentiality of 55 genes for bacterial conjugation of IncPα plasmids to yeast. Notably, a mutation in the *traJ* promoter region linked to improved bacterial conjugation offers researchers an excellent tool for initial DNA-delivery experiments, especially to yeasts with previously no reports of DNA transformation. It also highlights a critical role in the regulation of the *traJ* gene and the entire relaxosome operon, inciting investigation into its relationship to the eukaryotic recipient cells and associated co-culture toxicities. Future research should continue to improve the efficiency of bacterial conjugation to eukaryotic recipients and attempt to adapt this process for targeted DNA

delivery to diverse eukaryotes and specific subcellular localizations using superior conjugative plasmids. I found that adding an N-terminal yeast nuclear localization signal to pTA-Mob 2.0's relaxase (TraI) decreased the performance of bacterial conjugation 100–1000-fold. It will be for future researchers to use the improved conjugative plasmid, pSC5, to modify the specificity of this conjugative system for mitochondrial DNA delivery and expand mitochondrial transformation methods to additional species.

Finally, the progression towards a completed DBT cycle critical for synthetic biology will enable efficient production and delivery of designer mitochondrial genomes to an increasing number of eukaryotic hosts. Currently, biolistic-mediated gene gun delivery enables mitochondrial transformation for *S. cerevisiae*, *C. glabrata*, and *C. reinhardtii*. The installation of designer mitochondrial genomes could be attempted immediately using *S. cerevisiae* to determine the feasibility and current limitations preventing the installation of completely designer mitochondrial genomes. The state of the field is a nearly complete DBT test cycle for mitochondrial genomes of *S. cerevisiae* and *C. reinhardtii*. In order to expand the number of transformable organisms, selection markers and mitochondrial phenotypic knockouts must be characterized for adequately controlled experiments. It will then be possible to compare the biolistic-mediated gene gun delivery method of mitochondrial transformation to promising alternative methods being generated, such as bacterial conjugation. Once WGE tools for mitochondria are generated, genome-scale variation in the mitochondria can be studied, pathways that produce essential medicines or lucrative products for industry can be introduced, and these tools can be applied to additional chassis organisms.

5.6   References

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Appendices

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Appendix B: Supplemental Information for Chapter 2

B.1 Supplemental Tables

Table B-1. List of primers used to amplify and screen *Phaeodactylum tricornutum*'s mitochondrial genome.

Name	Primers	Length (bp)
Amplification of *P. tricornutum*'s mitochondrial genome: pPT-PCR		
Homology regions to the pAGE3.0 plasmid are highlighted		
Fragment 1	BK141F – ctgttacacgttggactgggacaaaatggtttattgaagg	5217
	BK141R – ttggaataaaagggttcgaacctttgaatgatcgtaccaa	
Fragment 2	BK142F – tttatttaacaaaatcgcctcttggggttcttatgcatca	5190
	BK142R – actagtcgttaaatggttagttagtttgtttttaataagc	
Fragment 3	BK143F – tggtcgggtaaaagacctacctggagtaaaatatcatcta	5197
	BK143R – gttttcccaaagatggggggcgatgtttttttccatttta	
Fragment 4	BK144F – gtgtaaatctatgaaaaagttattgaaatcaaaattacga	5175
	BK144R – tccatgtttttattaaaaatatttgaatgtttcagtatttt	
Fragment 5	BK145F – cccagtttcattttttgggctccaatctaaatgtcgttaaa	2815
	BK250R – cgacaagcacgagcgagatatcccaatcaagctagtatcgatcgcgcgcgggcgaagcttccctaaaaagatgacaaacc	
Fragment 6	BK251F – tttctgtaaatcggtttgtcatctttttagggaagcttcgcccgcgcgcgatcgatactagcttgattgggatatctcgc	9124
	BK88R – aagcttgaccgagagcaatcccgcagtcttcagtggtgtgatggtcgtctatgtgtaagtcaccaatgcactcaacgatt	
Fragment 7	BK88F – aatcgttgagtgcattggtgacttacacatagacgaccatcacaccactgaagactgcgggattgctctcggtcaagctt	8571
	BK245R – tcccccccccgcctaaggaaacttggtcgtaaaagcgtgaaaagcgatcgcgatcgtcttgccttgctcgtcggtgatgta	
Fragment 8	BK247F – tcgagctgtaagtacatcaccgacgagcaaggcaagacgatcgcgatcgctttcacgcttttacgaccaagtttcctta	5655
	BK146R – cttcacggaaaattcaatttcgctgagtttgtgctggaga	
Fragment 9	BK147F – gctgacgcctgcccagtgctgcaagattaaaggaaagggt	5299
	BK147R – agtaagcacggcgaaaaaaaaggtagaactggtaggagat	
Fragment 10	BK148F – tagcttttcgctccgaaacccaagatgttttttttcatc	9264
	BK140R – cccaacactattaaattcttcaactttgtttacaggattt	
Amplification of TAR cloning capture vector: pPT-TAR		
Homology regions to the pAGE3.0 plasmid are highlighted		
Fragment 1	BK92R – ttgcattttttgggcgttctttcattctagtatagcacctgcccatacgatggcgcgccatcgatactagcttgattgg	9142
	BK88 – aagcttgaccgagagcaatcccgcagtcttcagtggtgtgatggtcgtctatgtgtaagtcaccaatgcactcaacgatt	
Fragment 2	BK88F – aatcgttgagtgcattggtgacttacacatagacgaccatcacaccactgaagactgcgggattgctctcggtcaagctt	8581
	BK93F – gacagtgaaggtctttataaggcttattgctttaggcggccaaactcctccgcgtcgacggatcgtcttgccttgctcgt	
Diagnostic MPX PCR primers		
Amplicon 1	BK901F – tattgcatcgaggcacagag	265
	BK901R – gcccaaagcataggtgtcat	
Amplicon 2	BK902F – aaagctgcaaggcagttgat	171
	BK902R – aggccaaaaaggtttcgatt	
Amplicon 3	BK903F – ggcagaaaagctgagcctaa	224
	BK903R – cctatggttgcaaggcattt	
Amplicon 4	BK904F – ttcattttttgggctccaatc	334
	BK904R – cagtttcaggttcgggatgt	
Amplicon 5	BK905F – gttcttgtttcgccggatta	405
	BK905R – aacacagaccgacaccttcc	
Amplicon 6	BK906F – acgttttgcagtacccttgg	507
	BK906R – accataagtgcacgggaatc	

Table B-2. List of mutations identified in cloned *P. tricornutum* mitochondrial genomes. Plasmids containing the cloned genomes were sequenced using an Illumina MiSeq and mapped to their respective reference sequences by the CCIB DNA Core Facility at Massachusetts General Hospital. Mutations were identified using Geneious version 2020.0 created by Biomatters. Positions are based on residue numbering beginning with the first base in Fragment 1 of the result sequence and counting towards Fragment 2 (see Figure 3-1A). Published sequence: expected sequence as designed by combining published sequence for the *P. tricornutum* mitochondrial genome [1] and based on plasmid pAGE3.0 [2] according to the respective cloning strategy.

Position	Mutation	Location	Effect
pPT-PCR C1 | Reference: Published sequence			
8002	G > T	*mt-rps2*	$Arg_{114} >$ Ile
15,327	C > A	*mt-rpl2*	$Gln_{35} >$ Lys
16,259	1-bp insertion	*mt-rps19*	Frameshift
18,073	C > A	*mt-atp9*	$Leu_{42} >$ Met
22,687	G > A	*mt-cob* terminator	Unknown
22,714–22,871	158-bp insertion	*mt-cob* terminator	Unknown
34,783	129-bp deletion	Vector backbone (intergenic)	No effect
57,408	G > T	Intergenic	No effect
59,856	C > T	*mt-cox1*	$Thr_{245} >$ Ile
pPT-PCR C2 | Reference: Published sequence			
159	1-bp deletion	*mt-cox1* intron	Unknown
17,214	G > T	*mt-rps3*	$Met_{310} >$ Ile
27,716	G > T	Vector backbone (*S. meliloti repA2B2C2*)	$Ala_{135} >$ Glu
42,554	G > T	*mt-nad5*	$Glu_{595} >$ STOP
56,559	T > C	*mt-nad7*	$Ile_{191} >$ Thr
pPT-PCR C2-1 | Reference: pPT-PCR C2			
1197	C > T	*mt-cox1* intron	Unknown
1473	C > T	*mt-cox1* intron	Unknown
12,811	G > A	*mt-nad1*	$Asp_{295} >$ Asn
27,716	T > G	Vector backbone (*S. meliloti repA2B2C2*)	$Glu_{135} >$ Ala
55,673	G > A	*mt-cox2*	$Val_{195} >$ Ile
pPT-PCR C2-2 | Reference: pPT-PCR C2			
27,716	T > G	Vector backbone (*S. meliloti repA2B2C2*)	$Glu_{135} >$ Ala
pPT-TAR C1 | Reference: Published sequence			
17,871	C > A	*mt-cox3*	$Gly_{215} >$ Gly
pPT-TAR C2 | Reference: Published sequence			
8415	C > A	Vector backbone (*S. cerevisiae ARSH4*)	No effect
17,906	C > A	*mt-cox3*	$Gly_{215} >$ Gly
46,055	3847-bp deletion	Repeat region	Effect unknown.
46,182	8004-bp deletion	Repeat region	
46,235	T > C	Repeat region	Note: Mutations
46,246	403-bp deletion	Repeat region	detected in
46,300	A > G	Repeat region	repetitive region
46,304	C > T	Repeat region	are likely due to
46,442	G > A	Repeat region	sequencing errors.
46,536	G > A	Repeat region	
46,851	8165-bp deletion	Repeat region	
46,927	7906-bp deletion	Repeat region	
47,185	497-bp deletion	Repeat region	
47,757	3601-bp deletion	Repeat region	
47,891	C > T	Repeat region	
47,913	413-bp deletion	Repeat region	

Appendix C: Supplemental Information for Chapter 3

C.1 Supplemental Figures
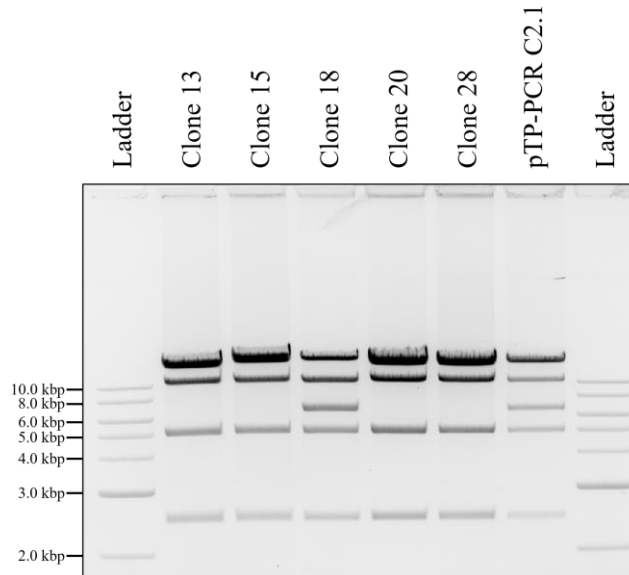
Figure C-1. PvuI restriction digest analysis of mutated mitochondrial genomes from the plasmid stability assay. pTP-PCR C2.1 (control) and five mutated clones (see Figure 3-4B) were analyzed. Band sizes of 6-, 2454-, 4863-, 6262-, 12,869-, 15,404-, and 15,749-bp are expected. Notes: 1 – Mutated pTP-PCR C2.1 genomes 13, 15, 20, and 28 show an incorrect restriction pattern confirming the result from the MPX PCR experiment (Figure 3-4B); 2 – The 6 bp band is not visible in this gel.
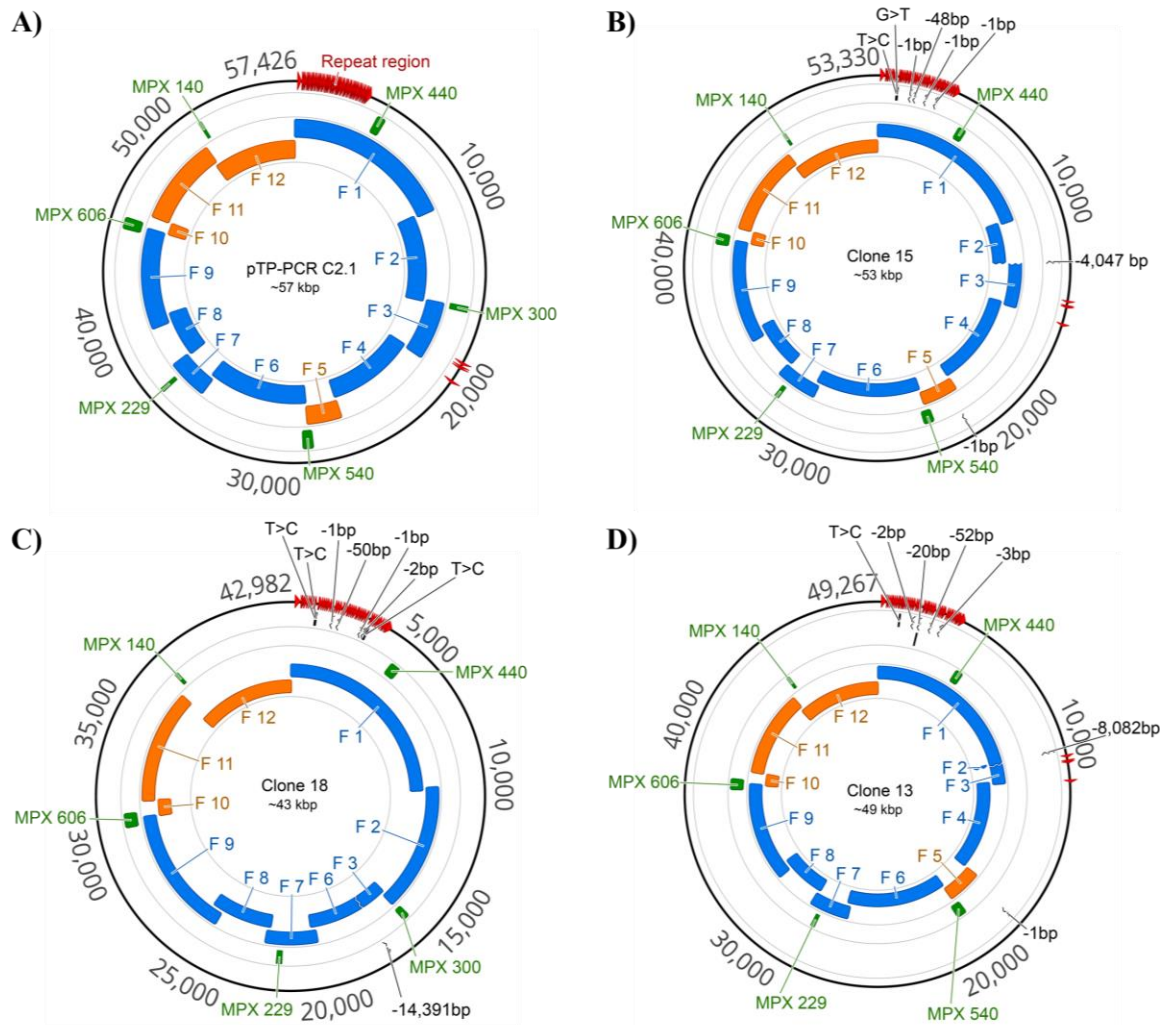
Figure C-2. Sequencing analysis of three mutated pTP-PCR C2.1 clones from the plasmid stability assay. A) Plasmid map of the reference sequence, pTP-PCR C2.1, prior to genome stability assay (Figure 3-4) experiment. **B–D)** Plasmid maps of clones 13, 15, and 18 with mutations identified after next-generation sequencing displayed. For all plasmid maps, the relative sizes and positions of the mitochondrial genome fragments (blue), plasmid backbone fragments (orange), and repetitive sequences (red) are shown. The six MPX PCR amplicons used for diagnostic screening and their sizes in bp are indicated (green). These images were generated using Geneious version 2020.2.4, created by Biomatters. Notes: 1 – in both **B)** and **D)** a deletion in fragments 2 and 3 resulted in the absent 300 bp MPX amplicon; 2 – in **C)** a deletion spanning fragments 3–6 resulted in the absent 540 bp MPX amplicon.

C.2 Supplemental Tables

Table C-1. List of primers used to amplify and screen *Thalassiosira pseudonana*'s mitochondrial genome.

Name	Primers	Length (bp)
Original amplification of the full *T. pseudonana* mitochondrial genome (pTP-PCR C1/2 – Design 1)		
Fragment 1	P1F – tttcattagttgcagtcactccgctttggtttggcgcgcctcgagcaacagggtacaacc P1R – agcgttaaaaagtttaaaaattagcgtatataaatttatg	10,735
Fragment 2	P2F – tatcttatacaatttttgctttcaggtaacattttattat P2R – aaaagaatttgcctgtattttaactacaaattataggaaa	6092
Fragment 3	P3F– aatctaaaactatcttacttaaaaaactaattcaaattaa P3R– ataaaataaaaaagtaagctgttcttaagcttgtttcggc	3610
Fragment 4	P4F – caggaatgttcaattttgttttaaacaaagtttaccaacttaatcctattatagaagata P4R – ggcctgcaacgttcgctaccttaggaccgttatagttacgtaaagtgttataaattctaa	6274
Fragment 5	P5F – tgaccaagatataaaatactttagaatttataacactttacgtaactataacggtcctaa P5R – ttaaatgtttaaatactgagtcgtttaaagttttgaccatctatattaccctgttatccc	2152
Fragment 6	P6F – tcgtttcatcgagttacgctagggataacagggtaatatagatggtcaaaactttaaacg P6R – ttcctttccaacaacacgaagcaaattttaataatcataagattatagtggataaatttag	7035
Fragment 7	P7F – aaaatttaaacatttacccactttctagtgatattcttgg P7R – attttgatgagctctggtttaaaatcttttaaaacattgc	2512
Fragment 8	P8F – tggttacgatactttgtataaaaaaaattcgagggatgga P8R – cgatttattgataagcaacgagattggtaaattaggccac	3250
Fragment 9	P9F – ccatactcatttgaatgtttcgagtttataaatttgccaaaagtactaagttttaggtca P9R – cgctataatgaccccgaagcagggttatgcagcggaagatctcgaggcagttcagattaa	6216
Fragment 10	P10F – cataaaaacagttcaaataattaatctgaactgcctcgagatcttccgctgcataaccct P10R – catagacggccgccagcccagccggcgagggcaaccagctcgcgatcgcgatcgtcttgcc	859
Fragment 11	P11F – agtacatcaccgacgagcaaggcaagacgatcgcgatcgcgagctggttgccctcgccgc P11R – ccatctgctcatcatccagctcgccaaccagaacacgataatcactttcggtaagtgcag	5367
Fragment 12	P12F – tgaccaggagctgcttactgaggacgcactggatgatctcatcccttcttttctactgac P12R – aagtaaggttataaatcattggttgtaccctgttgctcgaggcgcgccaaaccaaagcgg	5870
Amplification of the *T. pseudonana* reduced mitochondrial genome (pTP-PCR C3/4 – Design 2)		
Fragment 1	P2F – tatcttatacaatttttgctttcaggtaacattttattat P2R – aaaagaatttgcctgtattttaactacaaattataggaaa	6092
Fragment 2	P3F – aatctaaaactatcttacttaaaaaactaattcaaattaa P3R – ataaaataaaaaagtaagctgttcttaagcttgtttcggc	3610
Fragment 3	P4F – caggaatgttcaattttgttttaaacaaagtttaccaacttaatcctattatagaagata P13R – tcgtttaaagttttgaccattaaagtgttataaattctaa	6254
Fragment 4	P13F – taataatcaatggattttaatttaaaaacctataaacgtt P6R – ttcctttccaacaacacgaagcaaattttaataatcataagattatagtggataaatttag	7174
Fragment 5	P17F – ataaaattacgaaaaagtactaccataccgtctagtcgct P8R – cgatttattgataagcaacgagattggtaaattaggccac	5417
Fragment 6	P14F – ccatactcatttgaatgtttcgagtttataaatttgccaa P14R – cctaaaagacatagcacgcgacaagcacgagcgagatatcccaatcaagctagtatcgattaagcttgtatagcttgata	6372
Fragment 7	P15F – cgagaataaataatattagtctttgtatttctcttatcaagctatacaagcttaatcgatactagcttgattgggatatc P15R – aagcttgaccgagagcaatcccgcagtcttcagtggtgtgatggtcgtctatgtgtaagtcaccaatgcactcaacgatt	9136
Fragment 8	P16F – aatcgttgagtgcattggtgacttacacatagacgaccatcacaccacctgaagactgcgggattgctctcggtcaagctt P16R – cctaaaaaaaaattttgagaataagtaatattagtctttgatcgttatttatcaaaaacgatcgtcttgccttgctcgt	8441
Fragment 9	P18F – gaggcacttcgagctgtaagtacatcaccgacgagcaaggcaagacgatcgtttttgataaataacgatcaaagactaat P1R – agcgttaaaaagtttaaaaattagcgtatataaatttatg	6810
Final amplification of the *T. pseudonana* mitochondrial genome (pTP-PCR Design 1)		
Fragment 1	P25F – aaaatgcattgggaaaaaggttaaatttaccccaacgaaa P25R – gtagaatataaggctgtggattatgccacagttttttgct	5138
Fragment 2	P26F – gtaaccaagtatgcagtcccaattgcgcagatttacctac P26R – cgttttttattaaatcttgcaatttactgcaaagcaaca	5244
Fragment 3	P27F – tttgtagtgttgctcttattttaaacaactttgcggtttt P27R – cgtcacttcccagatccgcgctttctctgtccttcctgtg	5230
Fragment 4	P28F – taaaaaggccgtaatatccagctgaacggtctggttatag P28R – gcttaagtagacttgaactactgaccttacgcttatcagg	6884
Fragment 5	P29F – gagggcatcttttatattataataattccccaacctcaa P29R – atgtgacgggcggtgtgtacaaagccaaggtacgtattca	5959
Fragment 6	P30F – tcttagttcggattgtaagctgcaactcgtttacatgaag P30R – ttaaagctatagctttcaatgctgaatgatttgaagaagg	5107

Fragment 7	P31F – aatagtgaaaattatgtttttcgaaaattctacagcaggt	5948
	P31R – tgttgtaaacttatttgatatttctttgtttgttgcattg	
Fragment 8	P32F – attgaatcgttttacatgctccaggaatgttcaattttgt	5391
	P32R – atatttattttattgttaaatatttggattttcccagcta	
Fragment 9	P33F – aatttagcttcgaaataaaacgctaagccttgaaaagata	5888
	P33R – agttgtctttctggaaatcgccatttggtcagtttttag	
Fragment 10	P34F – tacacaaaagggaaagttaacattttcgtaacaatgcaaa	6155
	P34R – catttactcatgagttatttggggtagattcgtgtgataa	
Fragment 11	P35F – agccataatctgttaaaatacgctttaaatttgagtggtt	4289
	P35R – tatgatatgatagaacattataaaacggtggaaagaggta	

T. pseudonana diagnostic MPX PCR primers

Amplicon 1	P19F – gctcacgacatcagtttgct	140
	P19R – tgctggcttttcaagttcct	
Amplicon 2	P20F – gcttaattcacgcttattgaaaa	229
	P20R – atggctttgaaggacatcca	
Amplicon 3	P21F – agttaaatctatagaaaatgcaaatttagttattaacagt	300
	P21R – aaaatagaatctttgaaaaagtttctcctgaagttttatga	
Amplicon 4	P22F – agttttaatttttttgctgctattgtttattttttagc	440
	P22R – cacctaaactaatgggtctaaaaatatatgatataagctc	
Amplicon 5 (only +IR)	P23F – tggctaccctgtggaacacctacatctgtattaacgaagc	540
	P23R – gttttgggaacaggaagtcatgtgttcaaatcacattatt	
Amplicon 6 (only +IR)	P24F – ggtcataagccatatattcagtagaatataaatgtactat	606
	P24R – agccggccagcctcgcagagcaggattcccgttgagcacc	

Table C-2. List of mutations identified in cloned *T. pseudonana* mitochondrial genomes by next-generation sequencing.

Position	Mutation	Location/Gene	Effect
pTP-PCR C1.1 (Reference: pTP-PCR Design 1)			
888	A > G	Repeat region	Unknown
902	G > A	Repeat region	Unknown
1000	A > G	Repeat region	Unknown
1587	28-bp deletion	Repeat region	Unknown
1879	28-bp deletion	Repeat region	Unknown
1880	G > C	Repeat region	Unknown
2112	9-bp deletion	Repeat region	Unknown
2890	19-bp deletion	Repeat region	Unknown
2968	T > C	Repeat region	Unknown
3032	19-bp deletion	Repeat region	Unknown
3221	A > G	Repeat region	Unknown
3623	T > C	Repeat region	Unknown
9841	G > A	*rrnS*	Unknown
21,950	G > T	*rps12*	Val_{87} > Phe
22,337	2-bp deletion	*rps7*	Frameshift
22,433	1-bp deletion	*rps7*	Frameshift
22,716	C > A	*rpl14*	Gln_{44} > Lys
25,274	G > A	*tatC*	Thr_{56} > Ile
30,179	1-bp deletion	*rps19*	Frameshift
33,603	T > C	*nad11*	Leu_{373} > Ser
35,514	G > A	*trnS(gct)*	Unknown
42,462	G > A	*orf718* (*cox1* intron)	No effect
46,617	2-bp deletion	Vector backbone (intergenic)	No effect
56,480	G > A	Vector backbone (intergenic)	No effect
pTP-PCR C2.1 (Reference: pTP-PCR Design 1)			
848	A > G	Repeat region	Unknown
862	G > A	Repeat region	Unknown
960	A > G	Repeat region	Unknown
1549	26-bp deletion	Repeat region	Unknown
1843	27-bp deletion	Repeat region	Unknown
1843	G > C	Repeat region	Unknown
2074	89-bp deletion	Repeat region	Unknown
2385	31-bp deletion	Repeat region	Unknown
2743	17-bp deletion	Repeat region	Unknown
2822	T > C	Repeat region	Unknown
2886	17-bp deletion	Repeat region	Unknown
3077	A > G	Repeat region	Unknown
3479	T > C	Repeat region	Unknown
9697	G > A	*rrnS*	Unknown
11,838	1-bp deletion	*cox3*	Frameshift
22,763	C > T	*rpl14*	Pro_{107} > Ser
27,402	1-bp insertion	Intergenic	No effect
29,153	1-bp deletion	*rpl2*	Frameshift
34,681	1-bp deletion	Intergenic	No effect
41,218	G > A	*orf718* (*cox1* intron)	Pro_{388} > Ser
44,615	G > A	*nad5*	No effect
46,475	2-bp deletion	Vector backbone (intergenic)	No effect
49,206	1-bp insertion	Vector backbone (intergenic)	No effect

pTP-PCR C3.1 (Reference: pTP-PCR Design 2)

1846	1-bp deletion	Vector backbone (*S. meliloti repA2B2C2*)	Frameshift
7209	G > T	Vector backbone (*E. coli repE*)	Pro_{103} > Ser
21,050	1-bp insertion	*rrnL*	Unknown
23,484	G > A	*rrnS*	Unknown
24,048	G > A	*nad6*	Met_{142} > Ile
24,115	T > A	*nad6*	Leu_{165} > Met
34,435	C > G	*rps4*	Ile_{162} > Met
35,699	1-bp deletion	*rps12*	Frameshift
39,198	1-bp deletion	Intergenic	No effect
40,103	1-bp deletion	*rps11*	Frameshift
45,855	1-bp insertion	*nad11*	Frameshift
46,604	10,890-bp deletion	Several coding regions	Unknown

pTP-PCR C4.1 (Reference: pTP-PCR Design 2)

7–8	AC > TA	Vector backbone (intergenic)	No effect
748	C > T	Vector backbone (*nat*)	Pro_{50} > Leu
2513	C > T	Vector backbone (*S. meliloti repA2B2C2*)	Arg_{176} > His
11,179	G > A	Vector backbone (*E.coli parB*)	No effect
11,228	C > A	Vector backbone (*E. coli parB*)	Ala_{146} > Asp
18,322	1-bp deletion	*atp6*	Frameshift
23,483	G > A	*rrnS*	Unknown
25,862	C > T	*cox3*	No effect
33,411	5-bp deletion	Intergenic	No effect
41,950	C > T	*rps3*	Ser_{64} > Leu
42,081	1-bp deletion	*rps3*	Frameshift
46,598	G > A	Intergenic	No effect

Table C-3. Count of raw RNA sequencing reads for strains with either the pPT-PCR C2.1 genome or plasmid backbone alone (pPtGE31). Raw counts were enumerated by mapping against the appropriate reference and counted using HTSeq using –nonunique all mode. Three biological replicates were performed (repA, repB, repC) for each condition. Genes regions were counted for the features.

Gene	pPtGE31 repA	pPtGE31 repB	pPtGE31 repC	pPT-PCR C2.1 repA	pPT-PCR C2.1 repB	pPT-PCR C2.1 repC
cat	2224	4737	3625	3395	4988	6646
atp6	0	0	0	13	6	18
atp8	0	0	0	0	0	0
atp9	0	0	0	0	0	0
cob	0	0	0	51	82	133
cox1	0	0	0	1417	1576	3028
cox2	0	0	0	4	6	23
cox3	0	0	0	12	15	23
nad1	0	0	0	7	9	11
nad11-a	0	0	0	2	6	7
nad11-b	0	0	0	8	8	10
nad2	0	0	0	25	54	51
nad3	0	0	0	1	0	3
nad4	0	0	0	8	10	27
nad4L	0	0	0	0	0	0
nad5	0	0	0	11	17	17
nad6	0	0	0	9	4	17
nad7	0	0	0	29	32	52
nad9-rps14	0	0	0	4	7	11
rRNA 1.1	0	0	0	8	19	22
rRNA 1.2	0	0	0	81	99	165
rRNA 1.3	0	0	0	6	7	12
rRNA 1.4	0	0	0	38	78	145
rpl14	0	0	0	2	2	1
rpl16	0	0	0	0	2	3
rpl2	0	0	0	2	0	3
rpl5	0	0	0	0	0	0
rpl6	0	0	0	0	2	3
rps10	0	0	0	1	0	3
rps11	0	0	0	0	0	4
rps12	0	0	0	0	1	1
rps13	0	0	0	1	0	0
rps19	0	0	0	1	0	4

rps2	0	0	0	0	1	2
rps3	0	0	0	2	2	5
rps4	0	0	0	0	1	7
rps7	0	0	0	1	1	2
rps8	0	0	0	1	0	5
tatC	0	0	0	11	9	38
trnA(ugc)	0	0	0	1	0	0
trnC(gca)	0	0	0	0	1	0
trnD(guc)	0	0	0	0	0	0
trnE(uuc)	0	0	0	19	33	42
trnF(gaa)	0	0	0	1	0	4
trnG(gcc)	0	0	0	0	0	0
trnH(gug)	0	0	0	0	0	0
trnI(cau)	0	0	0	2	0	0
trnI(gau)	0	0	0	2	5	6
trnK(uuu)	0	0	0	0	0	0
trnL(uaa)	0	0	0	0	0	0
trnL(uag)	0	0	0	0	0	1
trnM(cau)	0	0	0	16	26	48
trnM(cau)	0	0	0	1	2	14
trnN(guu)	0	0	0	1	0	1
trnP(ugg)	0	0	0	0	0	0
trnQ(uug)	0	0	0	9	5	14
trnR(ucg)	0	0	0	0	0	1
trnR(ucu)	0	0	0	0	1	5
trnS(gct)	0	0	0	6	15	32
trnS(tga)	0	0	0	0	0	0
trnV(uac)	0	0	0	54	81	121
trnW(cca)	0	0	0	0	0	0
trnY(gua)	0	0	0	0	0	2

Table C-4. Count of raw RNA sequencing reads for strains with either the pTP-PCR C2.1 genome or plasmid backbone alone (pPtGE31). Raw counts were enumerated by mapping against the appropriate reference and counted using HTSeq using –nonunique all mode. Three biological replicates were performed (repA, repB, repC) for each condition. Genes regions were counted for the features.

Genes	pPtGE31 repA	pPtGE31 repB	pPtGE31 repC	pTP-PCR C2.1 repA	pTP-PCR C2.1 repB	pTP-PCR C2.1 repC
cat	2224	4737	3625	1856	1439	4337
atp6	0	0	0	0	0	1
atp8	0	0	0	0	0	0
atp9	0	0	0	0	0	1
cob	0	0	0	10	3	13
cox1	0	0	0	427	463	914
orf718	0	0	0	402	438	883
cox2	0	0	0	2	0	3
cox3	0	0	0	11	12	27
nad1	0	0	0	2	0	2
nad11	0	0	0	2	0	2
nad2	0	0	0	0	1	1
nad3	0	0	0	1	0	1
nad4	0	0	0	1	0	3
nad4L	0	0	0	0	0	0
nad5	0	0	0	5	2	5
nad6	0	0	0	1	0	6
nad7	0	0	0	46	27	38
nad9	0	0	0	0	0	0
rpl14	0	0	0	0	0	6
rpl16	0	0	0	0	5	1
rpl2	0	0	0	1	12	8
rpl5	0	0	0	0	0	1
rpl6	0	0	0	0	0	0
rps10	0	0	0	0	0	0
rps11	0	0	0	0	0	0
rps12	0	0	0	4	0	0
rps13	0	0	0	1	0	1
rps14	0	0	0	0	0	0
rps19	0	0	0	1	9	3
rps2	0	0	0	0	0	0
rps3	0	0	0	1	0	0
rps4	0	0	0	0	0	0

rps7	0	0	0	0	0	1
rps8	0	0	0	0	0	0
rrnS	0	0	0	23	17	36
rrnL	0	0	0	70	46	112
tatC	0	0	0	2	0	1
trnA(ugc)	0	0	0	1	0	0
trnC(gca)	0	0	0	0	0	0
trnD(guc)	0	0	0	0	0	0
trnE(uuc)	0	0	0	0	0	2
trnF(gaa)	0	0	0	0	0	0
trnG(gcc)	0	0	0	0	0	1
trnH(gug)	0	0	0	0	0	0
trnI(cau)	0	0	0	2	0	0
trnI(gau)	0	0	0	0	0	0
trnK(uuu)	0	0	0	0	0	1
trnL(uaa)	0	0	0	0	0	0
trnL(uag)	0	0	0	1	0	0
trnM(cau)	0	0	0	7	4	15
trnN(guu)	0	0	0	1	0	0
trnP(ugg)	0	0	0	0	0	0
trnQ(uug)	0	0	0	0	0	1
trnR(ucg)	0	0	0	0	0	1
trnR(ucu)	0	0	0	0	0	0
trnS(gct)	0	0	0	1	0	2
trnS(tga)	0	0	0	1	0	0
trnV(uac)	0	0	0	0	0	1
trnW(cca)	0	0	0	1	0	1
trnW(uca)	0	0	0	0	0	1
trnY(gua)	0	0	0	0	0	0

C.3 Supplemental Notes

Note C-1. Determination of outliers in the calculation of doubling time for *E. coli* strains. The median of the dataset was calculated, as well as the lower (Q1) and upper (Q3) quartiles representing the data points at which 25% of the data falls below and above, respectively. The interquartile range (IQR = Q3 - Q1) that indicates the boundaries of non-outlier data points was then calculated. Next, the inner fence of the dataset was found by multiplying IQR by 1.5, then subtracting that value from Q1 and adding it to Q3. Any point outside the inner fence is considered a minor outlier. The outer fence of the dataset was found by multiplying IQR by 3, then subtracting that value from Q1 and adding it to Q3. Any data point outside the outer fence is considered a major outlier. Here, we have only omitted major outliers from our determination of td.

Appendix D: Supplemental Information for Chapter 4

D.1 Supplemental Figures
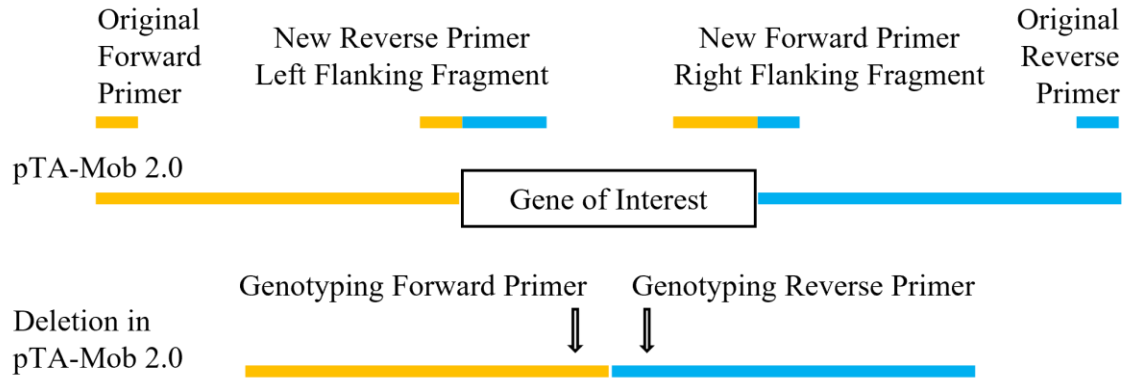
Figure D-1. Deletion plasmid assembly strategy. Each deletion plasmid was assembled from nine standard fragments as described in (Soltysiak et al. 2019) and two modified fragments amplified with original forward primer and new reverse primer, and new forward primer and original reverse primer. After assembly, each deletion was genotyped by MPX PCR.
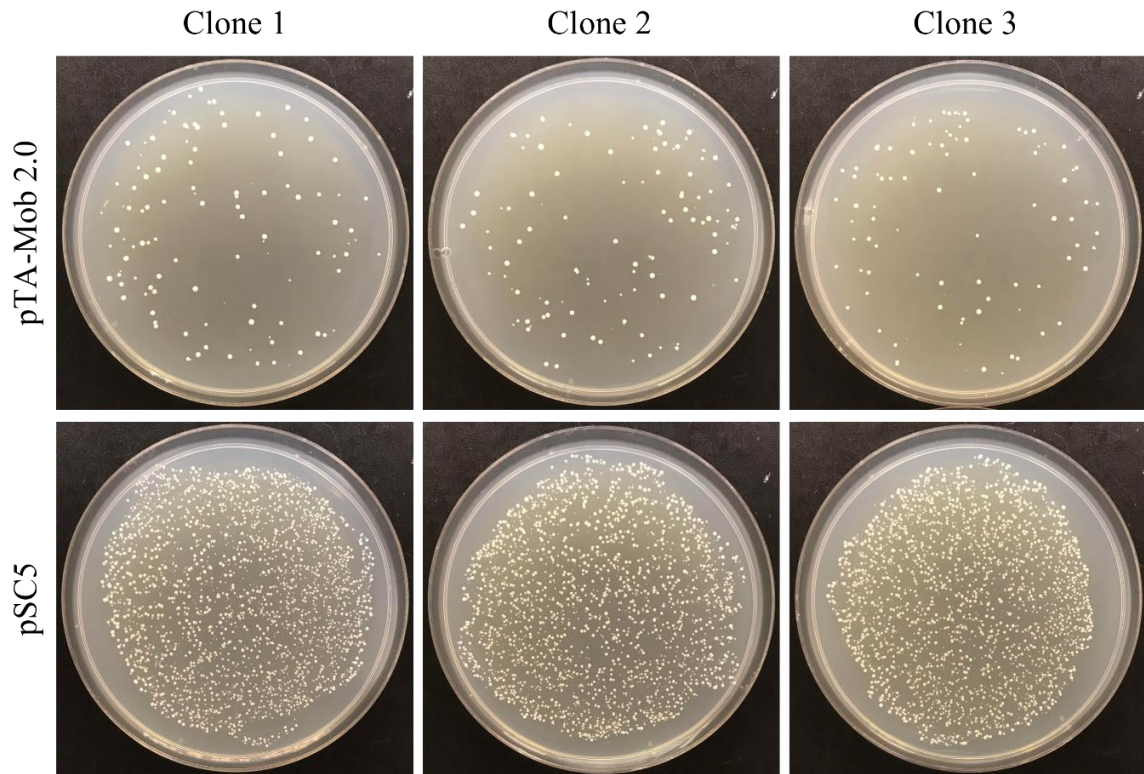
Figure D-2. Bacterial conjugation from *S. meliloti* to *S. cerevisiae*. Representative plates of yeast transconjugants following bacterial conjugation from three *S. meliloti* clones harboring either pTA-Mob 2.0 or pSC5, plated on synthetic complete media lacking histidine supplemented with ampicillin (100 µg mL^{-1}).
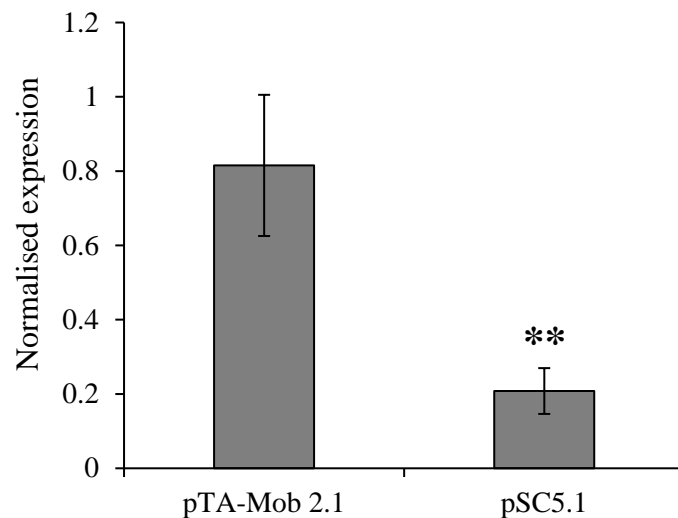
Figure D-3. Quantitative real-time polymerase chain reaction (qRT-PCR) of *traJ* expression. *traJ* mRNA expression in *E. coli* harboring the conjugative plasmids pTA-Mob 2.1 and pSC5.1 by qRT-PCR. The mean ± SE is given for six biological replicates normalized to the reference genes *rrsA* and *cysG*. Student's *t*-test was used to compare pTA-Mob 2.1 and pSC5.1, and asterisks denote the significant difference between the pairwise comparison (*, $p < 0.05$; **, $p < 0.01$).
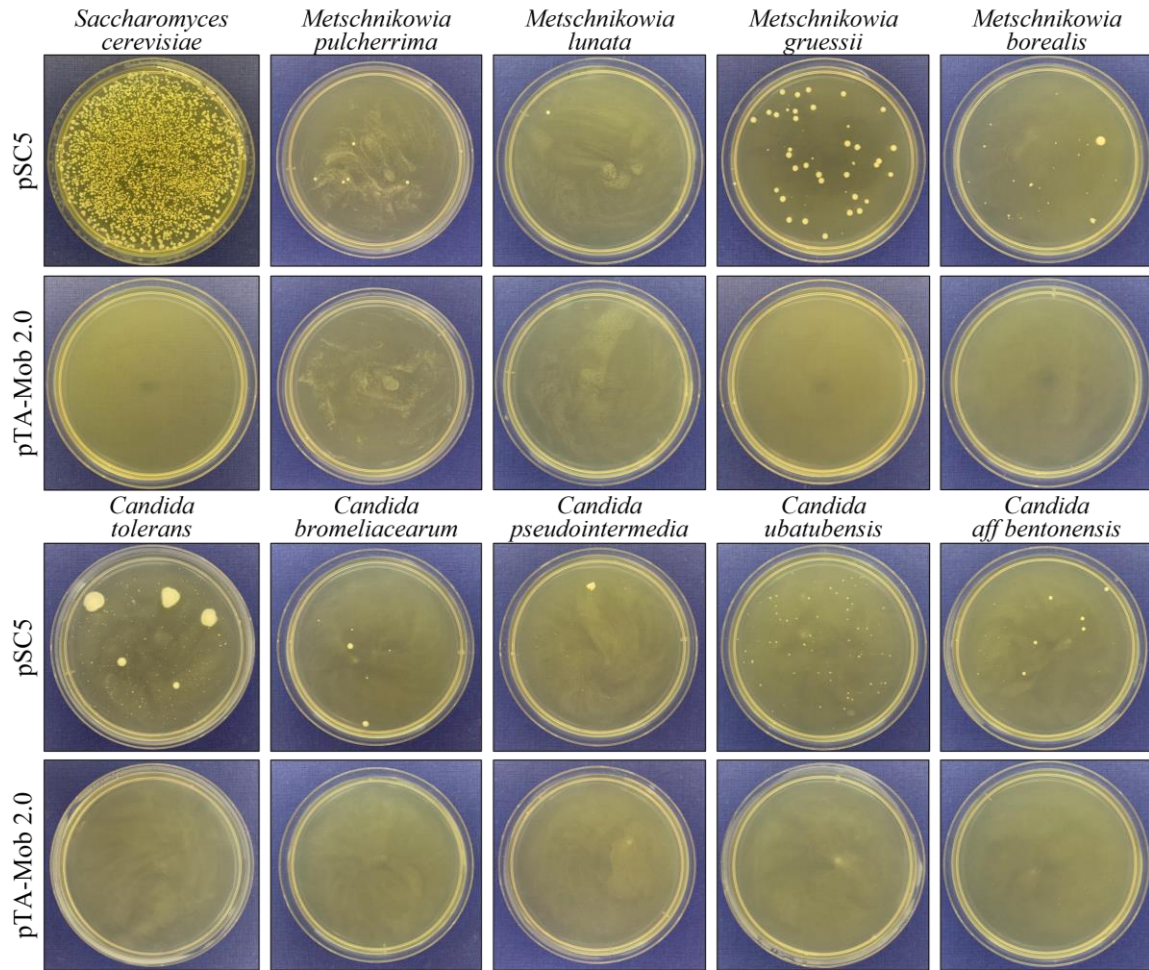
Figure D-4. Bacterial conjugation from *E. coli* to diverse yeast species.
Representative plates of yeast transconjugants following bacterial conjugation from *E. coli* harboring either pSC5 or pTA-Mob 2.0, plated on YPAD media supplemented with nourseothricin (100 µg mL^{-1}) and ampicillin (100 µg mL^{-1}).
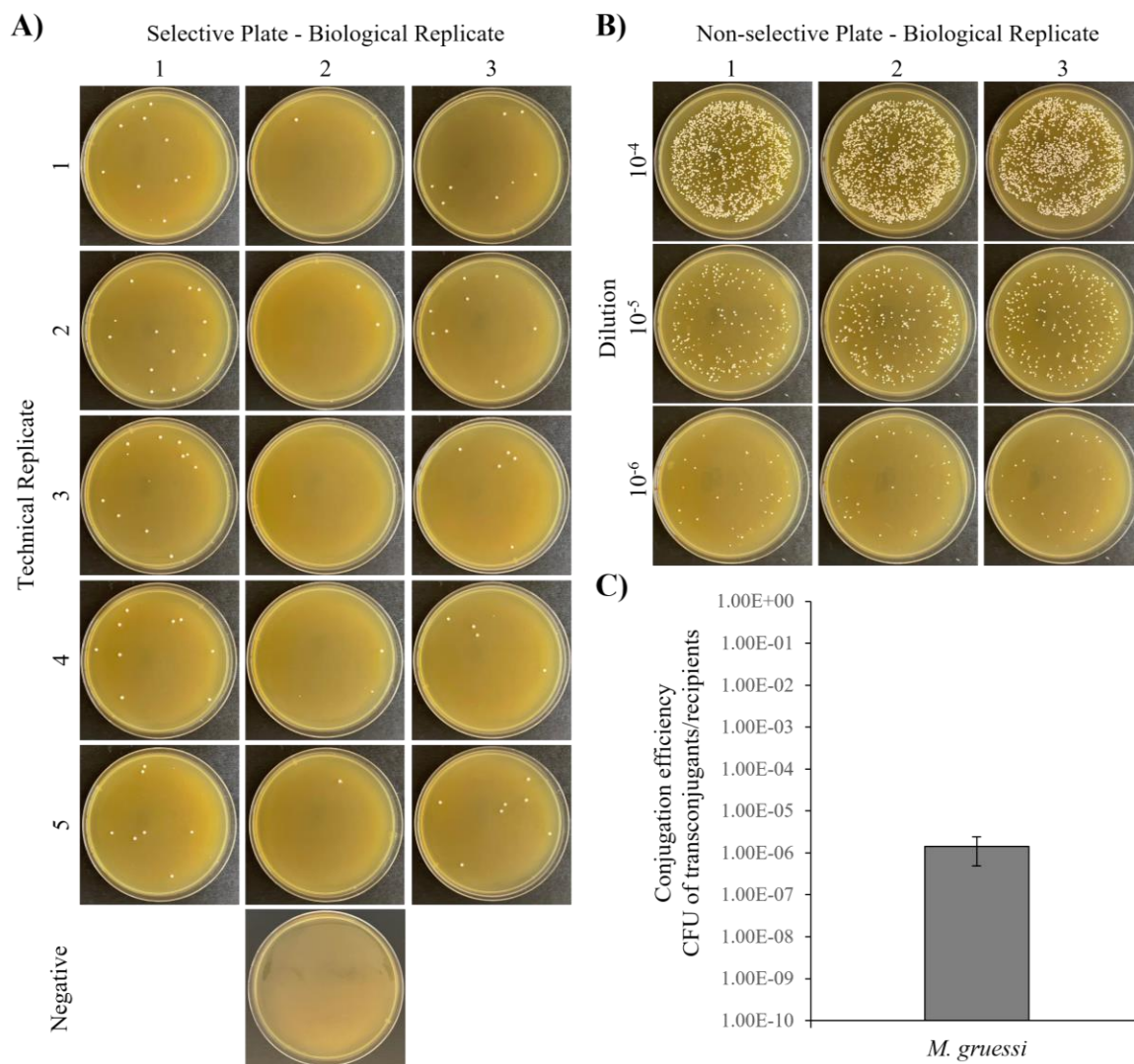
Figure D-5. Bacterial conjugation efficiency of pSC5 to *Metschnikowia gruessi*. A) Three replicates of *M. gruessi* transconjugants plated on five YPDA plates supplemented with nourseothricin (100 µg mL⁻¹) and ampicillin (100 µg mL⁻¹). **B)** Dilution series (10⁻³– 10⁻⁵) of *M. gruessi* plated on YPDA plates supplemented with ampicillin (100 µg mL⁻¹). **C)** Average bacterial conjugation efficiency of *M. gruessi* represents the mean ± standard deviation for three biological replicates.
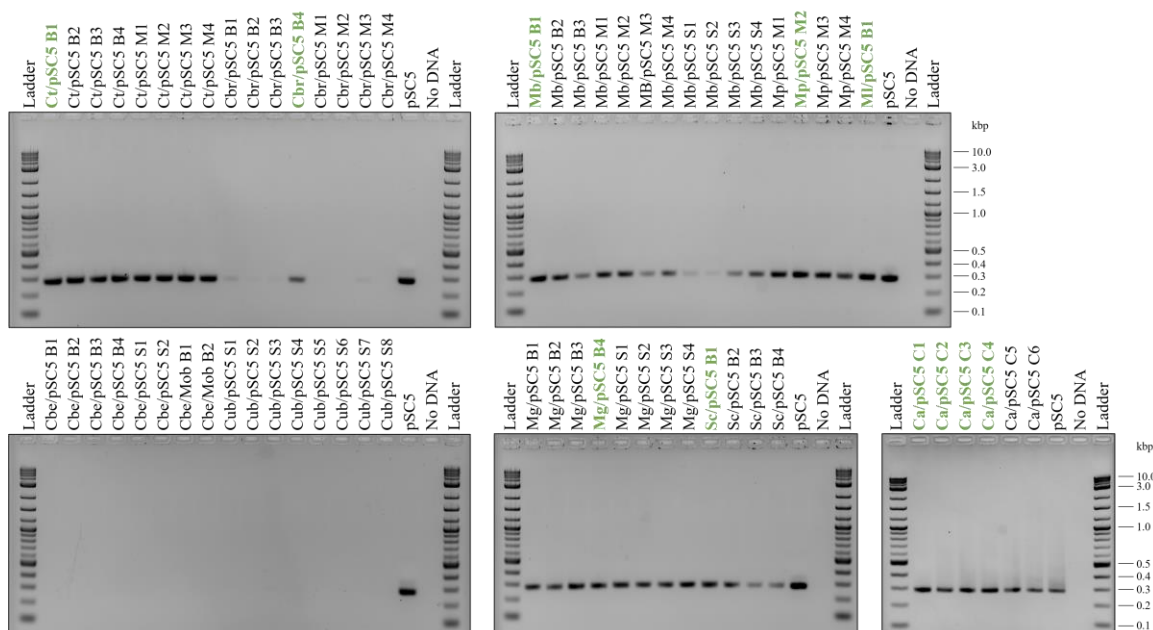
Figure D-6. Genotyping transconjugants of diverse yeast species. Genotyping of diverse yeast strains following bacterial conjugation with *E. coli* harboring pSC5. MPX PCR was performed to amplify the nourseothricin resistance gene of amplicon size 283-bp. Ct – *Candida tolerans*, Cbr – *Candida bromeliacearum*, Mb – *Metschnikowia borealis*, Mp – *Metschnikowia pulcherrima*, Ml – *Metschnikowia lunata*, Cbe – *Candida* aff. *bentonensis*, Cub – *Candida ubatubensis*, Mg – *Metschnikowia gruessi*, Sc – *Saccharomyces cerevisiae,* Ca – *Candida auris*, B – big colony, M – medium colony, and S – small colony. Clones highlighted in green were further analyzed with restriction enzyme digest and a phenotypic bacterial conjugation screen (Figure 4-5, Supplemental Figure D-7). Ladder – NEB 2-log ladder.
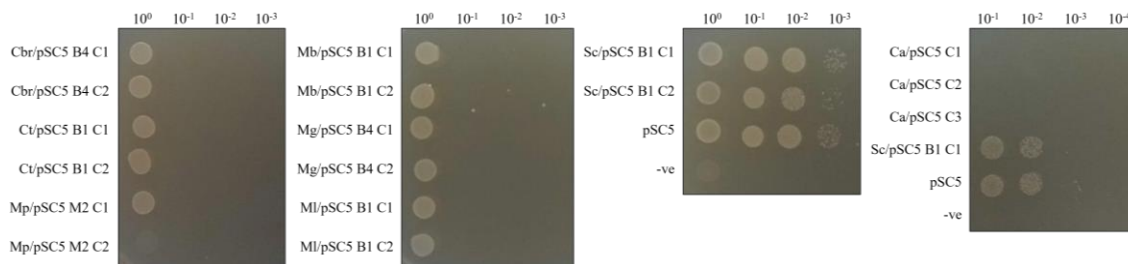
Figure D-7. Phenotypic bacterial conjugation screen (*E. coli* to *E. coli*) of recovered transconjugant plasmids from diverse yeast species. Selected recovered pSC5 plasmids from diverse yeast and *S. cerevisiae* were transformed into *E. coli* and tested for bacterial conjugation to *E. coli* harboring pAGE1.0. Transconjugant *E. coli* were spot plated on LB plates supplemented with chloramphenicol (30 µg mL^{-1}) and gentamicin (60 µg mL^{-1}). Ct – *Candida tolerans*, Cbr – *Candida bromeliacearum*, Cbe – *Candida bentonensis*, Cub – *Candida ubatubensis*, Mb – *Metschnikowia borealis*, Mp – *Metschnikowia pulcherrima*, Ml – *Metschnikowia lunata*, Mg – *Metschnikowia gruessi*, Sc – *S. cerevisiae,* Ca – *Candida auris*, B – Big colony, M – Medium colony, -ve – Negative (*S. cerevisiae* only).

D.2 Supplemental Tables

Table D-1. Description of pTA-Mob 2.0 deletion plasmid library. The fragment split by PCR amplification is listed, and regions of pTA-Mob 2.0 deleted are reported with respect to the 3' end of the backbone insertion re-indexed to position 1. Diagnostic MPX PCR primers used to screen deletion plasmids, and their respective amplicon sizes are shown. Genes either entirely or partially removed from pTA-Mob 2.0 in each deletion plasmid are listed, their location within pTA-Mob 2.0 is annotated in parentheses, and the primers used to remove them are provided.

Deletion plasmid	Fragment split	Region deleted	MPX primers	Amplicon (bp)	Gene(s) deleted Or partially deleted	Additional primers for splitting the fragment of interest. Top primer pairs with the original reverse primer for the fragment of interest. Bottom primer pairs with the original forward primer of the fragment of interest.
1	2	574-1241	F - aggcggtaaaggtgagcag R - gaagcctgcgaagagttgc	301	*upf16.5* (574-1241)	gcggcagagatgaacacgaccatcagcggctgcacagcgccattgacccaggcgtgttcc tgcgaggcagccggcctggtggaacacgcctgggtcaatggcgctgtgcagccgctgatg
2	2	1216-2364	F - ctctgtttatcggcagttcg R - gtattcgtgcagggcaagat	327	*trfA1* (1216-2364) & *trfA2* (1216-2073)	tggctgctgaacccccagccggaactgaccccacaaggcctcaccctccttgcgggattg tgccccggcgtgagtcggggcaatcccgcaaggagggtgaggccttgtggggtcagttcc
3	2	1216-2073	F - ctctgtttatcggcagttcg R - tcgatggtccagcaagctac	306	*trfA2* (1216-2073) & *trfA1* (1216-2364)	tggctgctgaacccccagccggaactgaccccacaaggccagttcctcgcgtgtcgatgg aggtttggcgaagtcgatgaccatcgacacgcgaggaactggccttgtggggtcagttcc
4	2	2938-3249	F - ctcggtgtcacgggtaagat R - cacgacgtagggggttctgat	393	*trbA* (2884-3249)	attttttcaccaacatccttcgtctgctcgatgagcggggccgccaaggggttagggcttgc tagcggctaaagaaggaagtgcaagcccctaacccttggccggccccgctcatcgagcagac
5	2	3583-4479	F - tgcgctttgacagttgtttt R - ttcgtcagccagctctcata	374	*trbB* (3520-4479)	gaccatcaaggagcgggccaagcgcaagctggaacgcgacggagtatttccaatgacaac tcagacggaacggaacagccgttgtcattggaaatactccgtcgcgttccagcttgcgct
6	2	4492-4929	F - attcaccgaaacccattgag R - catgattcggaacgcataga	349	*trbC* (4492-4929)	cggccagtacatcaccaaaaccctgtaaggagtatttccatcatggctctgcgcacgatc ttgcctgcgcgacggatggggatcgtgcgcagagccatgatggaaatactccttacaggg
7	2	4932-5239	F - ttcttccgaaccctgatctt R - tccttggaacgatgcttttt	304	*trbD* (4932-5243)	atgccgtgcgtgcggtaacggctggacggctcgcctaatcatgatccaagcaattgcgat cgccgaggcccgcgattgcaatcgcaattgcttggatcatgattaggcgagccgtccagc
8	2/3	5244-7794	F - gtcggtctgatcctgtggtt R - gtctgcttttcgcacacaac	338	*trbE* (5240-7798)	ccgcgagaacaccaatagccaagggaagcaataccgatgaatgagttttgcagacacgat tcttgaagatcaagccctgatcgtgtctgcaaaactcattcatcgtattgcttccctt
9	3	7799-8553	F - cgtctctacgacctggcact R - gtaccccgcctcccacttct	352	*trbF* (7795-8553)	ccggggcctcgccctgatgaatacctggaggcagcatgaggcactgaattatgaaaaag aggaccaaagcaaacagttccttttttcataattcagtgcctcatgctgcctccaggtatt
10	3	8565-9458	F - ggtcagcaccgaaatcactt R - agctgccgtacttggaggt	319	*trbG* (8565-9458)	gggacttctcctggtcgagactctgtgaggcactgaattacatgcgtaagattctgac tggccgcgagtgcgatgacggtcagaatcttacgcatggtaattcagtgcctcacagaag
11	3	9462-9944	F - acggcaagaaaaccatcatc R - tgaggtacatcggcatgttg	331	*trbH* (9462-9944)	cagccaggaccgcgtgaccatttcaaggggggaactaaaccgccaatgagcgaagatcaaa tggcgatgcgtccggtgccatttgatcttcgctcattggcggtttagttcccccttgaaa
12	3	9949-11,340	F - agcaacctgtatcgcctgac R - tcctcgatttgcttctggac	313	*trbI* (9949-11,340)	atggtcccgcgccgcgcatgggtcggaaggagtaagccactccaaggagtaacttatga aacattcttagcgagctcttcataagttactccttggagtggcttactccttccgaacc
13	3/4	11,357-12,133	F - gcagatgatcgccaaaaact R - gggcacttcttcaacagctc	312	*trbJ* (11,357-12,133)	ccctaccaggcgtttgactattaactccaaggagtaacttggggaggcgcgatgaagaaa gcaactgcgatgaagttgatttcttcatcgcgcctccccaagttactccttggagttaa
14	4	12,145-12,354	F - caaatccgtggccttctg R - acggtcaaggtccagaacag	349	*trbK* (12,145-12,354)	accgcgcaagcccgtctaagacctggtgaggggaggcgcgtgacgtatgaaaatccagac ccgcgagcgcggcagctctagtctgatttcatacgtcacgcgcctcccctcaccaggt
15	4	12,361-13,947	F - gatctgccgaaggtcacg R - cttgtggatgccgaagtacc	329	*trbL* (12,361-13,947)	aggcttcaagcccagcgaaaagaaagatggtgatgacgtaacgacttaggagctacg gcttttttcagttgcatggtcgtagctcctaagagtcgttacgtcatcaccactctttct
16	4	13,971-14,570	F - gttcgacgacaacagccttt R - aagaatgtttggccggaatct	324	*trbM* (13,971-14,570)	agcccgccccaatcctgaaacgactccttaggagctacgaccgggggaggagatgacgatgcc gtgccagcagcttggcaaacggcatcgctatcccctccccggtcgtagctcctaagagtc
17	4	14,586-15,290	F - gccatgcctataccgacttc R - tgcccagtaccagaagatca	336	*trbN* (14,586-15,290)	cgcctaccgtcggttcgacgcggtaaggggaggggatagcgggaggaacggccgtttagcg gaatgcccataggctttagccgctaaacggccgttcctcccgctatcccctccccttacc
18	4	15,321-15,584	F - ggcagctcatcatcaacaac R - ccaggacgaaaacgaaaaga	300	*trbO* (15,321-15,584)	cacgccataaggaggaacggccgtttagccgctaaagcctcgccctgcagggcgttctta tcatgcctcccccttggagtaagaacgccctgcaggccgaggctttagccgctaaacgg
19	4	15,621-16,355	F - aagcgccgtttgatcttct R - catggggatgttcagcagt	321	*trbP* (15,621-16,355)	atagccgcctgcagggcgttcttactccaaggggggaggccctttgagattccaatatgc ggtgcatttcttgagcaattgcatattggaatctcaaagggccctcccccttggagtaag
20	4	16,372-16,791	F - acgtactacccgctgcatct R - gatagaacgcttcggtgtcc	343	*upf31.7* (16,372-16,791)	gcgggctacttgttttcacctgacctttgagattccaatggggagggcggcggatgctg aggaagccctcaaccgtgcagcatccgccgccctccccattggaatctcaaaggtcag
21	4/5	16,806-17,501	F - ataaccagctcgccatcaag R - cagcaccaggaacatcgtc	318	*fiwA* (16,806-17,501)	cgcttgacgatgcgaagttctactgaggggagggcggccggctacaaccgtgcgcaaggcg tacatacatcctccctaatgccgcttgcgcacggttgtagccgccgccctccccctcagta
22	5	17,537-18,199	F - cgagctgctgaacaaggttt R - gagccaggtcaaacgagtgt	303	*upf32.8* (17,537-18,199)	tctgactacaaccgtgcgcaaggcgcattagggaggatgtgaaaagccgggcactgcccg cgcagcagcaaaaataaagccgggcagtgcccgggcttttcacatctccctaatgcgcct
23	5	18,223-18,882	F - gactacaccgaggggggaaag R - gagaaggacaccgaccgtta	331	*parA1* (18,223-18,882) & *parA2* (18,223-18,846)	gggacgcgaaaaggtgagaaaagccgggcactgcccggctttcacggctcgacggcgtg tcggcctctggtccgatccgcacgcgcctgcgagccgtggaaagccgggcagtgcccggctt
24	5	18,223-18,846	F - gactacaccgaggggggaaag R - gtgggtcaacatggagctg	326	*parA2* (18,223-18,846) & *parA1* (18,223-18,882)	gggacgcgaaaaggtgagaaaagccgggcactgcccggctccttcgtccctccgtttgtt ggaaatggcgacgcgagagcaacaaccgagggacgaaggagccgggcagtgcccggctt
25	5	18,843-19,688	F - tctcctggcgttcaagattc R - tccgtcatgtcgattgtcag	217	*parB* (18,843-19,376) & *parC* (19,373-19,666) & *parA1* (18,223-18,882) & *parA2* (18,223-18,846)	tgctcgtccgtactggcgcgcaggtagatgcgggcgacctggcgcattacagcaatacgc gcgctaggcgcatttaaattgcgtattgctgtaatgcgccaggtcgcccgcatctacctg
26	5	19,373-19,666	F - tgcttgtctaccagcacgtc R - tccgtcatgtcgattgtcag	318	*parC* (19,373-19,666)	agcgccggcagcggccgcagcatggcgtagcttcggcgctttgggcctagtctagccggc gtattgctgtaatgcgccatgccggctagactaggcccaaagccgccgaagctacgccatg
27	5	19,817-20,068	F - catggcgcattacagcaata R - gggaaagagttcgctcatgt	304	*parD* (19,817-20,068) & *parE* (20,065-20,376)	tgtgctaatgtggttacgtgtattttatgggagttatccacgccctacatcctcacggct cgtagatcggcttcggcctcagccgtgaggatgtaggccgtggataacctccataaaata
28	6	23,490-24,286	F - cgaggttggtggtaatcgtt R - gccgccatagtgtagccagat	328	*istB* (23,489-24,286)	ggaacatcgaccactgagtgcctatgaggagctgttgtgacgaaagtatcttagcgggca accgttatttgccattttcatgcccgctaagatactttcgtcacaacagctcctcatagg
29	6	24,763-24,993	F - agactagccaccaccattgc R - acggagtagcagcagaaaa	328	*aphA* (24,763-24,993)	gaacgatattgatcgagaagagccctgcgcagccgctgccgggcgatgcccctcgacctc tcctgaacgcctccctgatcgaggtcgagggggcatcgccggcagcggctgcgaggact
30	6	25,077-25,367	F - tgcaatttcatctcctgctg R - cgcaacgtctaccagttcag	308	*traA* (25,077-25,367)	ctcacaaagaaagcccgggcaatgcccggcttttctgctgacgcctcctagatcgagcgc aaggccgagcagaaacgctcggcgctcgatctaggaggcgtcagcagaaaaacgggccat
31	6	25,377-25,815	F - gttgacgatgatgctgttggg R - gccaggtgagaagtgctgtc	347	*traB* (25,375-25,815)	gaaaatcgtgcgggtacgcctcgatgttcatacgcctcctacgctctagttctccttagt gccaacgccgcgagaacctactaagggagaactagagcgtaggaggcgtatagaacatcga
32	6/7	25,831-29,016	F - accttcatggtcagccattc R - gagttcgaccccggaagaag	314	*traC1* (25,831-29,016) & *traC2* (25,831-28,071)	gtgccccgatctgtactttgttcatacgctctagttctccagccaattacctcccgtcat cacgacgaccgcgggccgccaatgacgggagggtaattggctggagaactagagcgtatgaa
33	6	25,831-28,071	F - accttcatggtcagccattc R - atcaaggcgctacaagagga	308	*traC2* (25,831-28,071) & *traC1* (25,831-29,016)	gtgccccgatctgtactttgttcatacgctctagttctccagttgcccccctgccaggct aacgacccagcagccatcgagcctgcgcagggggcaactggagaactagagcgtatgaa
34	7	29,023-29,286	F - tgtaacgctcccggtagtc R - agtctccgagctgcacaagt	340	*traD* (29,023-29,286)	cctgctcgtggaacggctttttacctctgccatagccaacgctttcactcctggttggt acagcaaaggccgtaacggcaccaaccagagagtgaaagcgttgctatggcagaggtcaa
35	7	29,292-31,505	F - accaggtcaatgtcgctctc R - gccgaggttctgcttatgtc	342	*traE* (29,292-31,505)	aagtcgtcctgctgcacgtcttgggatcattcatcgcttatatcccctaccctcacca tcatcaggccggttctgacctggtgaggtaggggggatataagcgatgaatgatcccaag
36	7	31,520-32,049	F - tagtcctccgggtctagcaa R - gcgacatggtttgttgacgtc	345	*traF* (31,520-32,053)	gcgattacaaggcgttcaaattgcatatatcccccctaccctcatatcgtgatcccctccc gacggccaccgtcgaggaaggggagggggatcacgatatgagggtaggggggatatatgcaa
37	7	32,054-33,953	F - gcttttggtggttgttgacct R - tgattatgttggtgcgcgt	316	*traG* (32,050-33,957)	cgatggcgacgtacttggtgaggcgctggaagcggtcattcatctacctcctacctcggg gcgaggctcccttaaaactacccgaggtagagtagatgaataggccgcttccagcgcct
38	7/8	33,958-36,131	F - ttttccgcccgtatctgtg R - gtatccaacggcgtcagaat	505	*traH* (34,253-34,612) & *traI* (33,954-36,152)	cccgtatctgtggccccacggcgttgtttcggttcttcatggggacgtgcttggcaatca aacggccgggggtgcgcgtgattgccaagcacgctccccatgaagaaccgaaacaacgc
39	7	34,253-34,612	F - gcaccagaatctcgtctttt R - gtcaacggcacagcagagt	316	*traH* (34,253-34,612) & *traI* (33,954-36,152)	tcggcaacatattctcggccgccgcgatctgttcgggcgtgctgcttgctcctccttcgtcagt gagcgccgccgtcaagaactactgacgaaggagcaagcacagcccgaacagatcgcggcg

40	8	36,149-36,190	F - ctcggtcttgccttgctc R - gtatccaacggcgtcagaat	488	*traX* (36,149-36,190) *traI* (33,954-36,152) & *traJ* (36,187-36,558)	tcgctcttcttgatggagcgcatggggacgtgcttggcaaggctctgccctcgggcggac gcaaggtcatgatggcgtggtccgcccgagggcagagccttgccaagcacgtccccatg
41	8	36,187-36,558	F - gcgaagtcgctcttcttgat R - gctctttggcatcgtctctc	333	*traJ* (36,187-36,558) & *traX* (36,149-36,190)	aatcacgcgcaccccccggccgtttttagcggctaaaaaagccgcttgccgaattctgacg ttccttggtgtatccaacggcgtcagaattcggcaagcggcttttttagccgctaaaacg
42	8	36,796-37,199	F - tcgctataatgaccccgaag R - gttgcgcgagttaatttcgt	338	*traK* (36,796-37,200)	atgcaggaaattactgaactgaggggacaggcgagagacgatggcgaaaattcacatggt ccccgccccttgccctgcaaaaccatgtgaattttcgccatcgtctctcgcctgtcccctc
43	8	37,201-37,921	F - aacggggaaggtcaagttct R - tgatggtatgcaggatcagc	335	*traL* (37,200-37,925)	caccttcaacccaacaccggacaaaaaggatctactgtaaatgagcgaccagattgaaga ccgcaatctcccggatcagctcttcaatctggtcgctcatttacagtagatcctttttgt
44	8	37,926-38,359	F - ggcaagagctttgagcagat R - tgtcaaacaagcccagctaa	312	*traM* (37,922-38,359)	cggcctgtttgaacagctcgacgcggccggccgtgctatgaatcgcagaggcgcagatgaa gccccggcaacgccgggcttttcatctgcgcctctgcgattcatagcacggccgccgcgt
45	8	38,411-39,758	F - ggcgaaggtaatgaaggaca R - tgcgaaaagcatcacctatg	348	*upf54.4* (38,412-39,758)	gcagatgaaaaagcccggccgttgccgggcttgtttttgcggacgcctcccttttttagccg cgcactcgttagagttttagcggctaaaaaggaaggcgtccgcaaaaacaagcccggcaa
46	8/9	40,292-41,218	F - tggttctcggtctggacaat R - acctgccgttaagtcgagaa	305	*kfrA* (40,292-41,218)	ttgttccatctattttagtgaactgcgttcgatttatcaggttggctccggtaattggta ggtaagagtattattattcttaccaattaccggagccaacctgataaatcgaacgcagtt
47	9	41,932-42,444	F - ggatagcttgcaacatcagga R - acccggacaagctgaaaaag	295	*korF* (41,941-42,444)	cttgcaacatcaggagccgtttcttttgttcgtcagtcattcttcggtcctccttgtagc gccgcacagacaacggttccgctacaaggaggaccgaagaatgactgacgaacaaaagaa
48	9	44,843-45,792	F - ttggttgaacatagcggtga R - gttcagacgacgctcatcaa	313	*klaC* (44,843-45,796)	aaaacaaaagcccggaaaccgggctttcgtctcttgccgctcatacggactcctgttggg ccagcccgtgcgcgagctggcccaacaggagtccgtatgagcggcaagagacgaaagccc
49	9/10	45,793-46,875	F - ctggtcgctgaatgtcgat R - gctcatcaccaccaagaaca	392	*klaB* (45,793-46,929) & *klaC* (44,843-45,796)	aggccgcctacctgggcgaaaacatcggtgtttgttggcatgtcgaaggcgacgatagggg aacgacgcacgacgccaaggcccctatcgtcgccttcgacatgccacaaacaccgatgtt
50	10	46,947-47,720	F - catgtcgaaggcgacgatag R - ttcgatggatgtttgctttg	317	*klaA* (46,947-47,720)	tgcgtcgttttcagtgcgttcatagggtctcccgccgtgtcgatacaccctcgcggtgg tccatcgaaaagcaattaacccaccgcgagggtgtatcgacacggcgggagaaccctatg
51	10	47,843-48,157	F - gacagctcgttgagggaatc R - ggtactgaccgcactcacct	332	*kleF1* (47,843-48,157) & *kleF2* (47,843-48,013)	ccgctaaaatttggggacaggtcatttacagaaagccagctgctcaaagctccttgaagg ggagggtcaagcaagccggcccctcaaggagctttgagcagctggctttctgtaaatgac
52	10	47,839-48,013	F - caaaacctccccttcaatc R - tttgagcaatgccaagacag	318	*kleF2* (47,843-48,013) & *kleF1* (47,843-48,157)	atcggctgactggttcaatccgaggccgagggctggggcgctttctgtaaatgacctgt ctggacgaattgaacacgcatcgccgtggagggtcaagcaagcggcccccttcaaggagct
53	10	48,185-48,508	F - ctccggattgaaccagtacg R - aagtcaccaagtgggtcgag	341	*kleE* (48,185-48,508)	cctgtcttggcattgctcaaagctccttgaagggggccgcttgcttgaccctccacggcga ctggacgaattgaacacgcatcgccgtggagggtcaagcaagcggcccccttcaaggagct
54	10	48,620-48,838	F - cacgcctgggaacttgataa R - gaatgctattgccgagaagc	319	*kleD* (48,620-48,838)	atcgaggtcaagcgcccccggagaaatccggggcgtcatcccatcatcccctggcgtcagt ggcgaaattccgggccggtcactgacgccaggggatgatggggatgacgccccggatttct
55	10	48,854-49,084	F - ctcgacccacttggtgactt R - cagggggaaaggtgttttcaa	324	*kleC* (48,854-49,084)	agttcaccctatctctacttgcatcatcatcccctggcgcggattggcctccggtaatt gccgggtagattcccaggtcaattaccggaggccaatccgcgccaggggatgatgatgca
56	10	49,237-49,452	F - gcttctcggcaatagcattc R - actggtccacccaggaagtc	338	*kleB* (49,237-49,452)	gaggcgtcatgcttgaaaacacctttcccctggcgtgcaaggccctatctccttgagaga ggcccggctacggtcgggccctctctcaaggagataggggccttgcacgccgcaggggaaaggt
57	10	49,501-49,734	F - ggctaagggtcgaaatgga R - actttacgccaagggagagg	336	*kleA* (49,501-49,734)	ctccttgagagaggcccgaccgtagcccgggcctcgttccgcggggttgatcctccggttg gggcacttcgcccaggtcagcaaccggaggatcaaccccgcggaacgaggcccggctacg

Table D-2. List of primers used to amplify the assembly fragments and genotype the plasmids created in Chapter 4.

Fragment	Primer	Primer sequence (5' to 3')	Expected size (bp)	Template
M1				
F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	5782	pTA-Mob 2.0
	R	tttaacctacttcctttggttccgggggatctcgcgactc		
F2	F	atcgaagagaagcaggacga	6373	pTA-Mob 2.0
	R	tgctggtccatgaagatgaa		
F3	F	tcgagctgatgtttgacgac	6137	pTA-Mob 2.0
	R	ggacttgaggttgctctgct		
F4	F	gtggacattggtttcagcaa	3693	pTA-Mob 2.0
	R	gcgagaacctactaaggagaactagagcgtacgtgcttcgaaccactcggagggacggtt		
F5	F	aaccgtccctccgagtggttcgaagcacgtacgctctagttctccttagtaggttctcgc	3476	pTA-Mob 2.0
	R	aagcgatgaatgatcccaag		
F6	F	gagcaatggatagccgatgt	6206	pTA-Mob 2.0
	R	aagcgatgaatgatcccaag		
F7	F	tgtaacgcttcccggtagtc	6295	pTA-Mob 2.0
	R	cattgcaaagcgactgatgt		
F8	F	gatccgctccttgaactctg	6259	pTA-Mob 2.0
	R	aggcccttgccaatgaat		
F9	F	ttctttgaatgcgcgggcgtcctggtgagcgtagtccagc	6000	pTA-Mob 2.0
	R	cgttcccgcctgcccctgattggcccgctgatcgaccgct		
F10	F	aatgttgcaaggcgatcag	5745	pTA-Mob 2.0
	R	agccctcccgtatcgtagtt		
M2				
F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	5782	pTA-Mob 2.0
	R	tttaacctacttcctttggttccgggggatctcgcgactc		
F2	F	atcgaagagaagcaggacga	6373	pTA-Mob 2.0
	R	tgctggtccatgaagatgaa		
F3	F	tcgagctgatgtttgacgac	6137	pTA-Mob 2.0
	R	ggacttgaggttgctctgct		
F4	F	ggaccaggcgcagtccaccatcaacggcctgatgagcgcc	6000	pTA-Mob 2.0
	R	atcggcgtgaagcccaacagggcca		
F5	F	gtggacattggtttcagcaa	6273	pTA-Mob 2.0
	R	agctcatgcatcacaacagc		
F6	F	gagcaatggatagccgatgt	6206	pTA-Mob 2.0
	R	aagcgatgaatgatcccaag		
F7	F	tgtaacgcttcccggtagtc	6295	pTA-Mob 2.0
	R	cattgcaaagcgactgatgt		
F8	F	gatccgctccttgaactctg	6259	pTA-Mob 2.0
	R	aggcccttgccaatgaat		
F9	F	ttctttgaatgcgcgggcgtcctgg	4224	pTA-Mob 2.0
	R	tgaacacgcatcgccgtggagggtcaagcagcggcaagagacgaaagcccggtttccggg		
F10	F	cccggaaaccgggctttcgtctcttgccgctgcttgaccctccacggcgatgcgtgttca	2664	pTA-Mob 2.0
	R	agccctcccgtatcgtagtt		
M3				
F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	5782	pTA-Mob 2.0
	R	tttaacctacttcctttggttccgggggatctcgcgactc		
F2	F	atcgaagagaagcaggacga	6373	pTA-Mob 2.0
	R	tgctggtccatgaagatgaa		
F3	F	tcgagctgatgtttgacgac	6137	pTA-Mob 2.0
	R	ggacttgaggttgctctgct		
F4	F	ggaccaggcgcagtccaccatcaacggcctgatgagcgcc	6000	pTA-Mob 2.0
	R	atcggcgtgaagcccaacagggcca		
F5	F	gtggacattggtttcagcaa	3693	pTA-Mob 2.0
	R	gcgagaacctactaaggagaactagagcgtacgtgcttcgaaccactcggagggacggtt		
F6	F	aaccgtccctccgagtggttcgaagcacgtacgctctagttctccttagtaggttctcgc	3476	pTA-Mob 2.0
	R	aagcgatgaatgatcccaag		
F7	F	tgtaacgcttcccggtagtc	6295	pTA-Mob 2.0
	R	cattgcaaagcgactgatgt		
F8	F	gatccgctccttgaactctg	6259	pTA-Mob 2.0
	R	aggcccttgccaatgaat		
F9	F	ttctttgaatgcgcgggcgtcctggtgagcgtagtccagc	6000	pTA-Mob 2.0
	R	cgttcccgcctgcccctgattggcccgctgatcgaccgct		
F10	F	aatgttgcaaggcgatcag	5745	pTA-Mob 2.0
	R	agccctcccgtatcgtagtt		
M4				
F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	5782	pTA-Mob 2.0
	R	tttaacctacttcctttggttccgggggatctcgcgactc		
F2	F	atcgaagagaagcaggacga	6373	pTA-Mob 2.0
	R	tgctggtccatgaagatgaa		
F3	F	tcgagctgatgtttgacgac	6137	pTA-Mob 2.0
	R	ggacttgaggttgctctgct		

F4	F	ggaccaggcgcagtccaccatcaacggcctgatgagcgcc	6000	pTA-Mob 2.0
	R	atcggcgtgaagcccaacagggcca		
F5	F	gtggacattggtttcagcaa	3476	pTA-Mob 2.0
	R	ggttctgacctggtgagggtagggggatatacgtgcttcgaaccactcggagggacggtt		
F6	F	aaccgtccctccgagtggttcgaagcacgtatatccccctaccctcaccaggtcagaacc	3655	pTA-Mob 2.0
	R	tgtaacgcttcccggtagtc		
F7	F	gatccgctccttgaactctg	6259	pTA-Mob 2.0
	R	aggcccttgccaatgaat		
F8	F	ttctttgaatgcgcgggcgtcctggtgagcgtagtccagc	6000	pTA-Mob 2.0
	R	cgttcccgcctgcccctgattggcccgctgatcgaccgct		
F9	F	aatgttgcaaggcgatcag	5745	pTA-Mob 2.0
	R	agccctcccgtatcgtagtt		
M5				
F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	11,593	M3C1
	R	tgctggtccatgaagatgaa		
F2	F	tcgagctgatgtttgacgac	8884	M3C1
	R	tcatcaggccggttctgacctggtgagggtagggggatatcgctatccctccccttacc		
F3	F	ggtaagggggaggggatagcgatatcccctaccctcaccaggtcagaaccggcctgatga	3675	M3C1
	R	cattgcaaagcgactgatgt		
F4	F	gatccgctccttgaactctg	6253	M3C1
	R	aggcccttgccaatgaat		
F5	F	ttctttgaatgcgcgggcgtcctggtgagcgtagtccagc	10,447	M3C1
	R	agccctcccgtatcgtagtt		
M6				
F1	F	aaaacaaaagcccggaaaccgggctttcgtctcttgccgccggggttgatcctccggttg	12,900	M3C1
	R	tgctggtccatgaagatgaa		
F2	F	tcgagctgatgtttgacgac	11,800	M3C1
	R	tcatcaggccggttctgacctggtgagggtagggggatatcgctatccctccccttacc		
F3	F	ggtaagggggaggggatagcgatatcccctaccctcaccaggtcagaaccggcctgatga	3675	M3C1
	R	cattgcaaagcgactgatgt		
F4	F	gatccgctccttgaactctg	6253	M3C1
	R	aggcccttgccaatgaat		
F5	F	ttctttgaatgcgcgggcgtcctggtgagcgtagtccagc	4262	M3C1
	R	gggcacttcgcccaggtcagcaaccggaggatcaaccccggcggcaagagacgaaagccc		
M7				
F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	5132	M3C1
	R	tgccccggcgtgagtcggggcaatcccgcaaggagggtgaccgcttgccctcatctgtta		
F2	F	ctggccggctaccgccggcgtaacagatgagggcaagcggtcaccctccttgcgggattg	3819	M3C1
	R	tgctggtccatgaagatgaa		
F3	F	tcgagctgatgtttgacgac	8884	M3C1
	R	tcatcaggccggttctgacctggtgagggtagggggatatcgctatccctccccttacc		
F4	F	ggtaagggggaggggatagcgatatcccctaccctcaccaggtcagaaccggcctgatga	3675	M3C1
	R	cattgcaaagcgactgatgt		
F5	F	gatccgctccttgaactctg	6253	M3C1
	R	aggcccttgccaatgaat		
F6	F	ttctttgaatgcgcgggcgtcctggtgagcgtagtccagc	10,447	M3C1
	R	agccctcccgtatcgtagtt		
M8				
F1	F	aaaacaaaagcccggaaaccgggctttcgtctcttgccgccggggttgatcctccggttg	6610	M3C1
	R	tgccccggcgtgagtcggggcaatcccgcaaggagggtgaccgcttgccctcatctgtta		
F2	F	ctggccggctaccgccggcgtaacagatgagggcaagcggtcaccctccttgcgggattg	11,686	M3C1
	R	tgacctggtgagggtagggggatatcgctatccctccccggtcgtagctcctaagagtc		
F3	F	agcccgcccaatcctgaaacgactcttaggagctacgaccggggaggggatagcgatatc	3710	M3C1
	R	cattgcaaagcgactgatgt		
F4	F	gatccgctccttgaactctg	6253	M3C1
	R	aggcccttgccaatgaat		
F5	F	ttctttgaatgcgcgggcgtcctggtgagcgtagtccagc	4262	M3C1
	R	gggcacttcgcccaggtcagcaaccggaggatcaaccccggcggcaagagacgaaagccc		
M3C1_F1				
F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	11,593	M3C1
	R	tgctggtccatgaagatgaa		
F2	F	tcgagctgatgtttgacgac	11,800	M3C2
	R	atcggcgtgaagcccaacagggcca		
F3	F	gtggacattggtttcagcaa	6500	M3C2
	R	aagcgatgaatgatcccaag		
F4	F	tgtaacgcttcccggtagtc	11,797	M3C2
	R	aggcccttgccaatgaat		
F5	F	ttctttgaatgcgcgggcgtcctggtgagcgtagtccagc	10,447	M3C2
	R	agccctcccgtatcgtagtt		
M3C1_F2				
F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	11,593	M3C2
	R	tgctggtccatgaagatgaa		
F2	F	tcgagctgatgtttgacgac	11,800	M3C1
	R	atcggcgtgaagcccaacagggcca		
F3	F	gtggacattggtttcagcaa	6500	M3C2
	R	aagcgatgaatgatcccaag		
F4	F	tgtaacgcttcccggtagtc	11,797	M3C2

	R	aggcccttgccaatgaat		
F5	F	ttcttttgaatgcgcgggcgtcctggtgagcgtagtccagc	10,447	M3C2
	R	agccctcccgtatcgtagtt		

M3C1_F3

F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	11,593	M3C2
	R	tgctggtccatgaagatgaa		
F2	F	tcgagctgatgtttgacgac	11,800	M3C2
	R	atcggcgtgaagcccaacagggcca		
F3	F	gtggacattggtttcagcaa	6500	M3C1
	R	aagcgatgaatgatcccaag		
F4	F	tgtaacgcttcccggtagtc	11,797	M3C2
	R	aggcccttgccaatgaat		
F5	F	ttcttttgaatgcgcgggcgtcctggtgagcgtagtccagc	10,447	M3C2
	R	agccctcccgtatcgtagtt		

M3C1_F4

F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	11,593	M3C2
	R	tgctggtccatgaagatgaa		
F2	F	tcgagctgatgtttgacgac	11,800	M3C2
	R	atcggcgtgaagcccaacagggcca		
F3	F	gtggacattggtttcagcaa	6500	M3C2
	R	aagcgatgaatgatcccaag		
F4	F	tgtaacgcttcccggtagtc	11,797	M3C1
	R	aggcccttgccaatgaat		
F5	F	ttcttttgaatgcgcgggcgtcctggtgagcgtagtccagc	10,447	M3C2
	R	agccctcccgtatcgtagtt		

M3C1_F5

F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	11,593	M3C2
	R	tgctggtccatgaagatgaa		
F2	F	tcgagctgatgtttgacgac	11,800	M3C2
	R	atcggcgtgaagcccaacagggcca		
F3	F	gtggacattggtttcagcaa	6500	M3C2
	R	aagcgatgaatgatcccaag		
F4	F	tgtaacgcttcccggtagtc	11,797	M3C2
	R	aggcccttgccaatgaat		
F5	F	ttcttttgaatgcgcgggcgtcctggtgagcgtagtccagc	10,447	M3C1
	R	agccctcccgtatcgtagtt		

pTA-Mob 2.0 Tp

F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	5752	pTA-Mob 2.0
	R	tttaacctacttcctttggttccgggggatctcgcgactc		
F2	F	atcgaagagaagcaggacga	6374	pTA-Mob 2.0
	R	tgctggtccatgaagatgaa		
F3	F	tcgagctgatgtttgacgac	6137	pTA-Mob2.0
	R	ggacttgaggttgctctgct		
F4	F	ggaccaggcgcagtccaccatcaacggcctgatgagcgcc	6000	pTA-Mob 2.0
	R	atcggcgtgaagcccaacagggcca		
F5	F	gtggacattggtttcagcaa	6273	pTA-Mob 2.0
	R	agctcatgcatcacaacagc		
F6	F	gagcaatggatagccgatgt	6206	pTA-Mob 2.0
	R	aagcgatgaatgatcccaag		
F7	F	tgtaacgcttcccggtagtc	6295	pTA-Mob 2.0
	R	cattgcaaagcgactgatgt		
F8a	F	gatccgctccttgaactctg	1881	pTA-Mob 2.0
	R	atccaacggcgtcagccgagggcaagcggatggctgatgaaaccaagccaaccaggaagg		
F8b	F	ccttcctggttggcttggtttcatcagccatccgcttgccctcggctgacgccgttggat	4435	pTA-Mob 2.0
	R	aggcccttgccaatgaat		
F9	F	ttcttttgaatgcgcgggcgtcctggtgagcgtagtccagc	6000	pTA-Mob 2.0
	R	cgttcccgcctgcccctgattggcccgctgatcgaccgct		
F10	F	aatgttgcaaggcgatcag	5745	pTA-Mob 2.0
	R	agccctcccgtatcgtagtt		

pTA-Mob 2.0 To

F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	5752	pTA-Mob 2.0
	R	tttaacctacttcctttggttccgggggatctcgcgactc		
F2	F	atcgaagagaagcaggacga	6374	pTA-Mob 2.0
	R	tgctggtccatgaagatgaa		
F3	F	tcgagctgatgtttgacgac	6137	pTA-Mob2.0
	R	ggacttgaggttgctctgct		
F4	F	ggaccaggcgcagtccaccatcaacggcctgatgagcgcc	6000	pTA-Mob 2.0
	R	atcggcgtgaagcccaacagggcca		
F5	F	gtggacattggtttcagcaa	6273	pTA-Mob 2.0
	R	agctcatgcatcacaacagc		
F6	F	gagcaatggatagccgatgt	6206	pTA-Mob 2.0
	R	aagcgatgaatgatcccaag		
F7	F	tgtaacgcttcccggtagtc	6295	pTA-Mob 2.0
	R	cattgcaaagcgactgatgt		
F8a	F	gatccgctccttgaactctg	1821	pTA-Mob 2.0
	R	gcagcccacctatcaaggtgtactgccttccagacgaacgaagagcgattgaggaaaagg		
F8b	F	taggccgacaggctcatgccggccgccgccgcctttcctcaatcgctcttcgttcgtct	4526	pTA-Mob 2.0
	R	aggcccttgccaatgaat		

F9	F	ttctttgaatgcgcgggcgtcctggtgagcgtagtccagc	6000	pTA-Mob 2.0
	R	cgttcccgcctgcccctgattggcccgctgatcgaccgct		
F10	F	aatgttgcaaggcgatcag	5745	pTA-Mob 2.0
	R	agccctcccgtatcgtagtt		

Sequencing primers to check mutations in *traJ* region

F1	F	gtttcagcaggccgcccagg		
	R	cgctgcataaccctgcttcg		

pSC5

F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	7280	pTA-Mob 2.0-NAT
	R	tttaacctacttcctttggttccggggatctcgcgactc		
F2	F	atcgaagagaagcaggacga	6373	M3C1
	R	tgctggtccatgaagatgaa		
F3	F	tcgagctgatgtttgacgac	11,800	M3C1
	R	atcggcgtgaagcccaacagggcca		
F4	F	gtggacattggtttcagcaa	3692	M3C1
	R	caagcattgggttccgtatctaaccatgaccgtgcttcgaaccactcggagggacggttt		
F5	F	aaaccgtccctccgagtggttcgaagcacggtcatggttagatacggaaccaatgcttg	2573	pGMO1
	R	aggagaactagagcgtaattaccctgttatccctacaaacaccctttcaatgggcttga		
F6	F	cattgaaagggtgtttgtagggataacagggtaattacgctctagttctccttagtaggt	3477	M3C1
	R	aagcgatgaatgatcccaag		
F7	F	tgtaacgcttcccggtagtc	11,797	M3C1
	R	aggcccttgccaatgaat		
F8	F	ttctttgaatgcgcgggcgtcctggtgagcgtagtccagc	10,447	M3C1
	R	cgttcccgcctgcccctgattggcccgctgatcgaccgct		
F9	F	aatgttgcaaggcgatcag	5745	MV3C1
	R	agccctcccgtatcgtagtt		

pSC5GGv1

F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	6112	pSC5
	R	taattagcatttttcgctttattctgttgtcgagatcttctccttgcaggttcaacaact		
F2	F	aagatctcgacaacagaataaagcgaaaaatgctaataatgcactaacactcaggcctc (To domesticate BsaI site)	7196	pSC5
	R	tgctggtccatgaagatgaa		
F3	F	tcgagctgatgtttgacgac	6137	pSC5
	R	ggacttgaggttgctctgct		
F4	F	ggaccaggcgcagtccaccatcaacggcctgatgagcgcc	6000	pSC5
	R	atcggcgtgaagcccaacagggcca		
F5	F	gtggacattggtttcagcaa	11,926	pSC5
	R	caatgtctgatgcaatatggacaattggtttcttggtctcattaccctgttatccctaca		
F6	F	accttcgggtgggcctttctgcgtttataggtctcatgcttacgctctagttctccttag	1187	pSC5
	R	cgaattgaaacggagggcgacaagaagggcgagaagtcgggcttctacgtcggccacctc		
F7	F	cgacttctcgcccttcttgtcgccctccgtttcaattcggtgcttcttgccgtccatga (To domesticate BsaI site)	8239	pSC5
	R	cattgcaaagcgactgatgt		
F8	F	gatccgctccttgaactctg	6256	pSC5
	R	aggcccttgccaatgaat		
F9	F	aatgttgcaaggcgatcag	4928	pSC5
	R	agccctcccgtatcgtagtt		
F10	F	attgaaagggtgtttgtagggataacagggtaatgagaccaagaaaccaattgtccatat (To amplify *mrfp* Gene)	1276	pAGE2.0-i
	R	gagaacctactaaggagaactagagcgtaagcatgagacctataaacgcagaaaggccca (To amplify *mrfp* Gene)		

***Sh ble* fragment for GG assembly**

Sh ble gene	F	ggtctcagtaatatcaagcttg	1063	pRS32 (From Shapiro lab)
	R	taggtctcaagcaactggatggcg		

HindII fragment for GG assembly

HindII toxic gene	F	ggtctcagtaaggcgcgcccttggcagaacatatccatcgcgtccgccatctccagcagc	1618	pUC57 HindII plasmid (Synthesized vector)
	R	ggtctcagatttatcttcgtttcctgcaggtttttgttctgtgcagttgggttaagaata		

***A. laidlawii* (Al) toxic gene fragments for GG assembly**

1st half of Al toxic gene	F	ggtctcagtaaggtaatgcttggcatgttcatatagatggtttaacagatcattataaag	2072	*A. laidlawii* strain PG-8A DNA
	R	ggtctcatagtgcattaaatcctggaggcgttaatttacttctatgtgcttcaaaggctt		
ACT1 intron	F	ggtctcaactagtatgttctagcgcttgcaccatcccatttaactgtaagaagaattgca	327	*S. cerevisiae* total DNA
	R	ggtctcactaaacatataatatagcaacaaaaagaatgaagcaatcgatgtttagtacatg		
2nd half of Al toxic gene	F	ggtctcattagtctaatgaaggtttacttcaatatattacgcttcattaaatggtttaactg	1061	*A. laidlawii* strain PG-8A DNA
	R	ggtctcaagcaggaccataagaagtccgaaaaactattaatctgtccaaaatgtttaata		

Genotyping *nat* marker

nat gene	F	tccagttgatccaccattga	283	
	R	caaccacaaatgaccagcac		

pSC5GGv2

F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	4235	pSC5GGv1
	R	atatggacaattggtttcttggtctcattacatatatacacatgtatatatatcgtatgc		
F2	F	tgggcctttctgcgtttataggtctcatgctgtatacctatgaatgtcagtaagtatgta	3149	pSC5GGv1
	R	tttaacctacttcctttggttccggggatctcgcgactc		
F3	F	taggagtgcggttggaacgt	6177	pSC5GGv1
	R	tgctggtccatgaagatgaa		
F4	F	tcgagctgatgtttgacgac	6137	pSC5GGv1
	R	ggacttgaggttgctctgct		
F5	F	ggaccaggcgcagtccaccatcaacggcctgatgagcgcc	6000	pSC5GGv1

	R	atcggcgtgaagcccaacagggcca		
F6	F	gtggacattggtttcagcaa	6300	pSC5GGv1
	R	aggagaactagagcgtaattaccctgttatccctacaaacacccttcaatgggcttcga		
F7	F	cattgaaagggtgtttgtagggataacagggtaattacgctctagttctccttagtaggt	3512	pSC5GGv1
	R	aagcgatgaatgatcccaag		
F8	F	tgtaacgcttcccggtagtc	6295	pSC5GGv1
	R	cattgcaaagcgactgatgt		
F9	F	gatccgctccttgaactctg	6253	pSC5GGv1
	R	aggcccttgccaatgaat		
F10	F	ttctttgaatgcgcgggcgtcctggtgagcgtagtccagc	6000	pSC5GGv1
	R	cgttcccgcctgcccctgattggcccgctgatcgaccgct		
F11	F	aatgttgcaaggcgatcag	4928	pSC5GGv1
	R	agccctcccgtatcgtagtt		
F12	F	gcatacgatatatatacatgtgtatatatgtaatgagaccaagaaaccaattgtccatat (To amplify *mrfp* gene)	1276	pAGE2.0-i
	R	tacatacttactgacattcataggtatacagcatgagacctataaacgcagaaaggccca (To amplify *mrfp* gene)		
pTA-Mob 2.1				
F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	5259	pTA-Mob 2.0
	R	gggagtatctggctgggccaacgttccaaccgcactcctaccggccagcctcgcagagca		
F2	F	gcggtgctcaacgggaatcctgctctgcgaggctggccggtaggagtgcggttggaacgt	6177	pTA-Mob 2.0
	R	tgctggtccatgaagatgaa		
F3	F	tcgagctgatgtttgacgac	6137	pTA-Mob 2.0
	R	ggacttgaggttgctctgct		
F4	F	ggaccaggcgcagtccaccatcaacggcctgatgagcgcc	6000	pTA-Mob 2.0
	R	atcggcgtgaagcccaacagggcca		
F5	F	gtggacattggtttcagcaa	6289	pTA-Mob 2.0
	R	agctcatgcatcacaacagc		
F6	F	gagcaatggatagccgatgt	6204	pTA-Mob 2.0
	R	aagcgatgaatgatcccaag		
F7	F	tgtaacgcttcccggtagtc	6295	pTA-Mob 2.0
	R	cattgcaaagcgactgatgt		
F8	F	gatccgctccttgaactctg	6256	pTA-Mob 2.0
	R	aggcccttgccaatgaat		
F9	F	ttctttgaatgcgcgggcgtcctggtgagcgtagtccagc	6000	pTA-Mob 2.0
	R	cgttcccgcctgcccctgattggcccgctgatcgaccgct		
F10	F	aatgttgcaaggcgatcag	4928	pTA-Mob 2.0
	R	agccctcccgtatcgtagtt		
pSC5.1				
F1	F	tgccgccgcgcgcatggtcgtaatgggaccgatagcccgt	6757	pSC5
	R	cacgcgacaagcacgagcgagatatcccaatcaagctagtccggccagcctcgcagagca		
F2	F	gcggtgctcaacgggaatcctgctctgcgaggctggccggtaggagtgcggttggaacgt	6177	pSC5
	R	tgctggtccatgaagatgaa		
F3	F	tcgagctgatgtttgacgac	6137	pSC5
	R	ggacttgaggttgctctgct		
F4	F	ggaccaggcgcagtccaccatcaacggcctgatgagcgcc	6000	pSC5
	R	atcggcgtgaagcccaacagggcca		
F5	F	gtggacattggtttcagcaa	9812	pSC5
	R	aagcgatgaatgatcccaag		
F6	F	tgtaacgcttcccggtagtc	6295	pSC5
	R	cattgcaaagcgactgatgt		
F7	F	gatccgctccttgaactctg	6256	pSC5
	R	aggcccttgccaatgaat		
F8	F	ttctttgaatgcgcgggcgtcctggtgagcgtagtccagc	10,549	pSC5
	R	agccctcccgtatcgtagtt		
qRT-PCR primers				
rrsA gene	F	ctcttgccatcggatgtgccca	106	pTA-Mob 2.1 and pSC5.1
	R	ccagtgtggctggtcatcctctca		cDNA
cysG gene	F	ttgtcggcggtggtgatgtc	136	pTA-Mob 2.1 and pSC5.1
	R	atgcggtgaactgtggaataaacg		cDNA
traJ gene	F	acgacgcccgtgattttgtag	109	pTA-Mob 2.1 and pSC5.1
	R	gccttccagacgaacgaaga		cDNA

Table D-3. Bacterial conjugation phenotype of each plasmid in the pTA-Mob 2.0 deletion plasmid library. Bacterial conjugation phenotypes of pTA-Mob 2.0 deletion strains are displayed as the ratio of deletion plasmid transconjugants relative to pTA-Mob 2.0 transconjugants. All clones were tested using 1 biological and 3 technical replicates, except for deletion plasmid 32 C2*, where 6 biological and 1–3 technical replicates were used. Additional replicas were performed for plasmid 32 C2* due to the high variations in the initial experiments. Deletions were categorized as either non-essential (green; 0.51–5.98), semi-essential (light-green; 0.06–0.50), or essential (red & orange; 0–0.05) based on their bacterial conjugation ratio. Contradictory results for clones for the same genes could be due to mutations introduced during the PCR fragment amplification or yeast assembly. N/D: Not done.

Deletion plasmid	Clone name	Ratio for clone 1	Ratio for clone 2
1	C1, C2	0.36	1.04
2	C1, C5	1.25	0.00
3	C2, C3	1.90	0.00
4	C2, C3	0.00	0.00
5	C1, C2	0.00	0.00
6	C2, C3	0.00	0.00
7	C2, C3	0.00	0.00
8	C4, C5	0.00	0.00
9	C2, C8	0.00	0.00
10	C2, C2.1	0.00	0.00
11	C2, C3	0.00	0.00
12	C2, C5	0.00	0.00
13	C4, C5	0.00	0.00
14	C1, C2	2.37	3.60
15	C1, C5	0.00	0.00
16	C1, C5	3.09	0.01
17	C2, C3	N/D	0.12
18	C2, C3	1.31	1.11
19	C4, C5	N/D	0.15
20	C2, C3	0.54	0.39
21	C4, C5	0.31	0.98
22	C2, C3	0.53	0.64
23	C1, C2	0.51	0.46
24	C3, C16	0.63	1.03
25	C1, C2.1	N/D	N/D
26	C2.2, C3	0.52	1.39
27	C1, C2	2.06	0.11
28	C2	N/D	0.34
29	C2, C5	0.98	0.00
30	C6, C7	4.36	1.22
31	C1, C2	0.57	1.47
32	C1, **C2***	0.75	5.98*
33	C3, C6	0.20	0.24
34	C1, C3	0.00	0.51
35	C1.1, C6	0.33	1.09
36	C1, C2	0.00	0.00
37	C2	0.00	N/D
38	C2, C3	0.00	0.00
39	C2, C8	0.00	0.24
40	C1, C5	0.00	0.00
41	C5, C7	0.00	0.00
42	C2, C4	N/D	0.00
43	C2, C4	0.00	0.00
44	C3, C5	0.00	0.00
45	C9, C10	0.00	0.04
46	C1.1, C10	0.00	0.22
47	C1, C5	0.65	1.49
48	C1	0.05	N/D
49	C1, C5	0.32	0.41
50	C4, C5	0.39	0.67
51	C3, C5	N/D	N/D
52	C1, C2	1.81	0.61
53	C2	N/D	0.69
54	C1, C2	0.00	0.00
55	C1, C5	0.43	0.41
56	C2, C3	0.46	0.60
57	C1, C5	0.65	1.03

Table D-4. Whole plasmid sequencing of minimal conjugative plasmid 3 (M3C1 and M3C2). Mutations in M3C1 and M3C2 were identified by next-generation sequencing and aligned to the reference sequence: pTA-Mob 2.0. Nucleotide numbering begins at the forward primer of Fragment 1. Note: The 4912 bp deletion in M3C1 and M3C2 was removed intentionally.

Plasmid	Fragment	Plasmid position	Nucleotide mutation	Amino acid mutation	Gene
M3C1	1	3342	1 bp insertion (T)		
M3C1	2	14,111	$G_{731} > T$	$Arg_{244} > Leu$	*trbF*
M3C1	2	20,676	$G_{505} > T$	$Asp_{169} > Tyr$	*trbN*
M3C1	3	25,788	$C_{138} > A$		
M3C1	3	26,489	4,912 bp Deletion		*URA3*
M3C1	4	37,156	$T_{77} > G$	$Glu_{26} > Ala$	*traJ*
M3C1	4	37,242–37,246	GAATT > CTCGG		
M3C2	3	25,787	$C_{138} > A$		*parE*
M3C2	3	26,488	4,912 bp Deletion		*URA3*
M3C2	4	37,458	G > T		
M3C2	5	48,347	$A_{47} > G$	$Leu_{16} > Ser$	*klaA*

Table D-5. *Cis-* and *trans-***bacterial conjugations of pSC5.** *S. cerevisiae* transconjugant concentrations following bacterial conjugation of pSC5 compared to pTA-Mob 2.0 in either *cis-* (self-transmissible) or *trans-* (mobilization of a secondary plasmid – pAGE2.0.T) and *E. coli* transconjugant concentrations following bacterial conjugation of pSC5 compared to pTA-Mob 2.0 in *cis-* from *E. coli* (Figure 4-4). Results are shown as colony-forming units per mL (CFU mL^{-1}) for four biological replicates, each with two technical replicates.

Configuration	Plasmid	Rep 1 (CFU mL^{-1})	Rep 2 (CFU mL^{-1})	Rep 3 (CFU mL^{-1})	Rep 4 (CFU mL^{-1})	Average (CFU mL^{-1})
Cis (*S. cerevisiae*)	pTA-Mob 2.0	3.1×10^3	4.2×10^3	3.6×10^3	3.8×10^3	3.7×10^3
	pSC5	1.9×10^6	1.3×10^5	1.3×10^5	1.0×10^5	1.4×10^5
Trans (*S. cerevisiae*)	pTA-Mob 2.0	6.6×10^2	5.7×10^2	1.3×10^3	2.8×10^3	1.3×10^3
	pSC5	5.3×10^4	8.3×10^4	8.5×10^4	6.2×10^4	7.1×10^4
Cis (*E. coli*)	pTA-Mob 2.0	2.2×10^6	1.0×10^6	2.2×10^6	2.7×10^6	2.1×10^6
	pSC5	1.0×10^6	1.4×10^6	1.0×10^6	1.2×10^6	1.2×10^6

Table D-6. Recipient yeast cell concentrations used in bacterial conjugation experiments of Figure 4-3. The concentration of *S. cerevisiae* recipient cultures formed on the non-selective plate (1 x YPAD supplemented with ampicillin 100 µg mL^{-1}) following the bacterial conjugation of pSC5 and pTA-Mob 2.0 in *cis-* and *trans-* configuration. Concentrations are presented as colony-forming units per mL (CFU mL^{-1}) for four biological replicates, each with two technical replicates.

Configuration	Plasmid	Rep 1 (CFU mL^{-1})	Rep 2 (CFU mL^{-1})	Rep 3 (CFU mL^{-1})	Rep 4 (CFU mL^{-1})	Average (CFU mL^{-1})
Cis	pTA-Mob 2.0	1.3 x 10^7	1.5 x 10^7	3.9 x 10^7	4.7 x 10^7	2.9 x 10^7
	pSC5	6.7 x 10^7	6.9 x 10^7	1.1 x 10^8	1.1 x 10^8	9.1 x 10^7
Trans	pTA-Mob 2.0	8.0 x 10^6	4.0 x 10^6	4.3 x 10^7	3.7 x 10^7	2.3 x 10^7
	pSC5	1.2 x 10^7	1.5 x 10^7	9.5 x 10^7	9.3 x 10^7	5.4 x 10^7

Table D-7. *S. cerevisiae* **cell viability following bacterial conjugation with different** *E. coli* **strains.** *S. cerevisiae* cell concentrations obtained by hemocytometer (all cells) or plating on non-selective plates (1 x YPAD supplemented with ampicillin 100 µg mL^{-1}; live cells) following a 3 h incubation at 30 ⁰C alone or with *E. coli* either harboring no plasmid, pTA-Mob 2.0, or pSC5.

Donor Recipient	Hemocytometer cell count (cells mL^{-1})	Colony count (CFU mL^{-1})	
		Technical replicas	Average
Donor: no *E. coli* Recipient: *S. cerevisiae*	5.08 x 10^8	2.01 x 10^8	2.08 x 10^8
		2.27 x 10^8	
		1.97 x 10^8	
Donor: *E. coli* Epi300 Recipient: *S. cerevisiae*	6.14 x 10^8	2.05 x 10^8	2.19 x 10^8
		2.37 x 10^8	
		2.15 x 10^8	
Donor: *E. coli* Epi300 with pTA-Mob 2.0 Recipient: *S. cerevisiae*	2.56 x 10^8	1.4 x 10^7	1.63 x 10^7
		1.0 x 10^7	
		2.5 x 10^7	
Donor: *E. coli* Epi300 with pSC5 Recipient: *S. cerevisiae*	4.06 x 10^8	1.41 x 10^8	1.44 x 10^8
		1.45 x 10^8	
		1.47 x 10^8	

Table D-8. *S. cerevisiae* **cell viability following bacterial conjugation with different *S. meliloti* strains.** *S. cerevisiae* cell counts by hemocytometer (all cells) or plating on non-selective plates (1 x YPAD supplemented with ampicillin 100 µg mL^{-1}; live cells) following a 3 h incubation at 30 ºC alone or with *S. meliloti* either harboring pTA-Mob 2.0 or pSC5.

Donor Recipient	Hemocytometer cell count (cells mL^{-1})		Colony count (CFU mL^{-1})	
	Biological replicas	Average	Biological replicas	Average
Donor: no *S. meliloti* Recipient: *S. cerevisiae*	3.04×10^8	3.04×10^8	1.27×10^8	1.27×10^8
Donor: *S. meliloti* Rm4126 R$^-$ with pTA-Mob 2.0 Recipient: *S. cerevisiae*	3.16×10^8 3.48×10^8 2.58×10^8	3.07×10^8	9.70×10^7 1.10×10^8 4.70×10^7	8.47×10^7
Donor: *S. meliloti* Rm4126 R$^-$ with pSC5 Recipient: *S. cerevisiae*	3.84×10^8 4.52×10^8 3.98×10^8	4.11×10^8	1.85×10^8 1.67×10^8 1.27×10^8	1.60×10^8

Table D-9. *S. cerevisiae* **transconjugant colony count following bacterial conjugation with** *S. meliloti***.** Colony counts of *S. cerevisiae* transconjugant re-suspension (2 mL) following bacterial conjugation with three biological replicates of *S. meliloti* (Rm4126) harboring either pTA-Mob 2.0 or pSC5, plated on synthetic complete yeast media lacking histidine supplemented with ampicillin (100 µg mL^{-1}) (Supplemental Figure D-2).

| Plasmid | Colony count (CFU) | | | |
| | 100 µL | | 50 µL | |
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
pTA-Mob 2.0 #1	114	147	53	68
pTA-Mob 2.0 #2	108	123	34	54
pTA-Mob 2.0 #3	92	87	46	39
pSC5 #1	2514	1044	1341	546
pSC5 #2	2399	2145	1254	1159
pSC5 #3	2504	572	1220	260

Table D-10. Yeast transconjugant colony counts for the bacterial conjugation-based antifungal experiment (Figure 4-7). *S. cerevisiae* transconjugant colony counts following bacterial conjugation with *E. coli* harboring pAGE2.0.T and either pSC5, pSC5-toxic1, pSC5-toxic2, or pSC5-toxic3. Note: Colonies were counted from plating 100 µL of undiluted (1x) and diluted (10x) yeast transconjugant re-suspension (2 mL) on synthetic complete yeast media lacking either histidine (*HIS3*) or tryptophan (*TRP1*). Colonies were counted manually. tmtc – too many to count; Rep – replicate.

	Plasmid	Yeast selection marker (CFU)								Ratio
		HIS3				*TRP1*				
		Rep 1	Rep 2	Rep 3	Average	Rep 1	Rep 2	Rep 3	Average	*HIS3/TRP1*
1 x	pSC5	tmtc	tmtc	592	592.0	tmtc	tmtc	302	302.0	1.960
	pSC5-toxic1	tmtc	tmtc	230	230.0	tmtc	tmtc	264	264.0	0.871
	pSC5-toxic2	25	17	5	15.7	594	tmtc	169	381.5	0.041
	pSC5-toxic3	2	1	0	1.0	428	tmtc	188	308.0	0.003
10 x	pSC5	208	436	55	233	174	262	33	156.3	1.490
	pSC5-toxic1	62	105	27	64.7	107	149	23	93	0.695
	pSC5-toxic2	4	1	0	1.7	66	72	18	52	0.032
	pSC5-toxic3	0	0	0	0	50	107	17	58	0

D.3 Supplemental Notes

Note D-1. Bacterial conjugation from *E. coli* to *E. coli* – *cis*-configuration. *E. coli*, donor and recipient strains, were prepared as in Section 4.4.5, except the donor strains were resuspended in 5 mL of ice-cold 10% glycerol, and 500 µL aliquots were prepared in 1.5 mL Eppendorf tubes. To assess the bacterial conjugation of pSC5 between bacteria, two donor strains of *E. coli* harboring either pSC5 or pTA-Mob 2.0 and an *E. coli* recipient strain harboring pAGE1.0 (chloramphenicol 15 µg mL^{-1}; Brumwell et al. 2019; Table 1) were prepared and stored in the -80 °C freezer. On the day of the bacterial conjugation, conjugation plates (20 mL, LB media with 1.5% agar) were prepared, and tubes containing the *E. coli* strains were removed from the freezer and thawed on ice. Once thawed, 10 µL of the donor *E. coli* strain was added to 100 µL of the recipient *E. coli* strain and mixed by pipetting prior to being transferred to the plate and spread evenly. Plates were incubated at 30 °C for 90 min and then were scraped with 1.5 mL of sddH$_2$O and mixed thoroughly by vortexing for 5 s. A dilution series (10^{-1}–10^{-8}) was created in a 96-well plate, and 100 µL of dilutions 10^{-1}–10^{-4} were plated on selection plates (25 mL, LB media with 1.5% agar supplemented with chloramphenicol 15 µg mL^{-1} and gentamicin 40 µg mL^{-1}). On non-selective plates (LB media with 1.5% agar supplemented with chloramphenicol 15 µg mL^{-1}), 100 µL of dilutions 10^{-1}–10^{-8} were plated. Plates were incubated at 37°C overnight, and the following morning the colonies were counted, and bacterial conjugation efficiency was calculated (transconjugant CFU / recipient CFU).

Note D-2. Bacterial conjugation from *S. meliloti* to *S. cerevisiae* – *cis*-configuration. *S. meliloti* was prepared similarly to *E. coli*; overnight cultures of a single colony were diluted to OD$_{600}$ of 0.1 in 50 mL of LB media with appropriate antibiotics (streptomycin 100 µg mL^{-1} and gentamicin 40 µg mL^{-1}) and grown until an OD$_{600}$ of 1.0 was achieved. On the day of bacterial conjugation, the conjugation plates (20 mL, synthetic complete yeast media lacking histidine 1.8% agar and 10% LBmc media) were made, and the *S. meliloti* and *S. cerevisiae* cells were thawed on ice for ~20 min. Once thawed, 50 µL of *S.*

cerevisiae was added to 100 µL of *S. meliloti* and mixed by gentle pipetting before being transferred to the plate and spread evenly. The plates were incubated at 30°C for 3 h. Next, the plates were scraped with 2 mL of sddH$_2$O and mixed thoroughly by vortexing for 5 s. For each bacterial conjugation, 3 biological replicates and 1 technical replicate were used, and 100 µL of each dilution (10^0–10^{-1}) for each sample was plated on selection plates (25 mL, synthetic complete yeast media lacking histidine, 2% agar, supplemented with ampicillin (100 µg mL^{-1})). The plates were incubated at 30 °C, scored after 4 days, and bacterial conjugation efficiency was calculated.

Note D-3. Bacterial conjugation from *E. coli* to *S. cerevisiae* – c*is*- and t*rans*-configuration. To assess bacterial conjugation of pSC5 to *S. cerevisiae* in *cis*-orientation, two donor strains of *E. coli* harboring either pSC5 or pTA-Mob 2.0 (gentamycin 40 µg mL^{-1}) were prepared as in Section 4.4.5, and in *trans*-orientation two donor strains of *E. coli* harboring either pSC5 and pAGE2.0.T or pTA-Mob 2.0 and pAGE2.0.T (gentamycin 40 µg mL^{-1} and chloramphenicol 15 µg mL^{-1}) were prepared as in Section 4.4.5 and all stored in the -80°C freezer. On the day of bacterial conjugation, the conjugation plates (*cis* – 20 mL, synthetic complete yeast media lacking histidine 1.8% agar and 10% LB media; *trans* – 20 mL, synthetic complete yeast media lacking tryptophan 1.8% agar and 10% LB media) were prepared, and the *E. coli* and *S. cerevisiae* cells were thawed on the ice for ~20 min. Once thawed, 50 µL of *S. cerevisiae* was added into the *E. coli* tube containing 100 µL of cells and mixed by pipetting before being transferred to the plate and spread evenly. The plates were incubated at 30 °C for 3 h. Next, the plates were scraped with 2 mL of sddH$_2$O and mixed thoroughly by vortexing for 5 s. A dilution series (10^0–10^{-7}) was generated, and two technical replicates of 100 µL for dilutions 10^0 – 10^{-4} were plated on selection plates (*cis* – 25 mL, synthetic complete yeast media lacking histidine 2% agar supplemented with ampicillin (100 µg mL^{-1}); *trans* – 25 mL, synthetic complete yeast media lacking tryptophan 2% agar supplemented with ampicillin (100 µg mL^{-1})); and two technical replicates of 100 µL for dilutions for each sample (10^{-4}–10^{-7}) were plated on non-selective plates (25 mL, 1 x YPDA supplemented with ampicillin

(100 µg mL^{-1})). The plates were incubated at 30°C, scored after 4 days, and bacterial conjugation efficiency was calculated.

Note D-4. Bacterial conjugation from *E. coli* to diverse yeast and transconjugant analysis. Bacterial conjugation proceeded as described in Section 4.4.5, except once dried, the conjugation plates were incubated at 30°C for 12 h, and selection plates were incubated at 30°C for 3 days before the number of colonies was counted. The plasmid was isolated from selected diverse yeast transconjugants to test the recovery of the pSC5 plasmid. The recovered plasmids were transformed into *E. coli* by electroporation and re-conjugated back from *E. coli* to diverse yeast species following the protocol in Section 4.4.5, except on selective plates, cells were spot plated rather than spread on full plates. After bacterial conjugation, cells were scraped with 2 mL of sddH$_2$O. These cells were serially diluted in 96-well plates, and 5 µL of different dilutions (10^0–10^{-4}) were spot plated in 1 x YPDA media supplemented with nourseothricin (100 µg mL^{-1}).

Note D-5. Bacterial conjugation-based kill assay in *S. cerevisiae*. To assess yeast killing facilitated by bacterial conjugation, three donor *E. coli* strains harboring pAGE2.0.T and either pSC5-toxic1, pSC5-toxic2, or pSC5-toxic3 (gentamicin 40 µg mL^{-1} and chloramphenicol 15 µg mL^{-1}) and the recipient *S. cerevisiae* were prepared as in Section 4.4.5 and stored in the -80°C freezer. On the day of bacterial conjugation, the conjugation plates (20 mL, synthetic complete yeast media lacking histidine 1.8% agar and 10% LB media) were made, and the *E. coli* and *S. cerevisiae* cells were thawed on ice for ~20 min. Once thawed, 10 µL of *S. cerevisiae* was added into the *E. coli* tube containing 100 µL of cells and mixed by pipetting before being transferred to the plate and spread evenly. The plates were incubated at 30°C for 3 h. Next, the plates were scraped with 2 mL of sddH$_2$O and mixed thoroughly by vortexing for 5 s. For each bacterial conjugation, 4 biological replicates and 1 technical replicate were used, and 100 µL of each dilution (10^0 – 10^{-1}) for each sample was plated on both selective plates (25 mL, synthetic complete yeast media lacking histidine and synthetic complete yeast media lacking tryptophan, 2% agar, supplemented with ampicillin (100 µg mL^{-1}). The plates

were incubated at 30°C, colonies were scored after 4 days, and killing efficiency (CFU on synthetic complete yeast media lacking histidine / CFU on synthetic complete yeast media lacking tryptophan) was calculated.

Curriculum Vitae

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EDUCATION

PhD Candidate, Biochemistry   2017 – Present
University of Western Ontario, London, ON

Bachelor of Medical Sciences, Honors Specialization   2012 – 2017
in Interdisciplinary Medical Sciences
University of Western Ontario, London, ON

SCHOLARSHIPS AND AWARDS

NSF RCN Travel Award, Build-A-Cell Workshop #8	$800	2022
The Canadian Graduate Scholarship – Doctoral (CGS-D)	$105,000	2021
Ontario Graduate Scholarship	$15,000	2020
NSF RCN Travel Award, SynCell2020	$750	2020
NSF RCN Travel Award, Build-A-Cell Workshop #6	$400	2020
Harold B. Stewart Memorial Lecture and Research Showcase, 2nd Place Poster Presentation	$20	2018
Symposium on Synthetic Biology 2.0, 2nd Place Poster Presentation	$150	2017
The Western Scholarship of Excellence	$2000	2013

PUBLICATIONS

Cochrane, RR, Shrestha, A, Severo de Almeida, MM, Agyare-Tabbi, M, Brumwell, SL, … Karas, BJ (2022). Superior conjugative plasmids delivered by bacteria to diverse fungi. BioDesign, 9802168.

Cochrane, R.R., Brumwell, S.L., Shrestha, A., Giguere, D.J., Hamadache, S., Gloor, G.B., Edgell, D.R. and Karas, B.J. (2020) Cloning of *Thalassiosira pseudonana's* mitochondrial genome in *Saccharomyces cerevisiae* and *Escherichia coli*. *Biology*, 9(11), 358.

Cochrane, R.R., Brumwell, S.L., Soltysiak, M.P., Hamadache, S., Davis, J.G., … Karas, B.J. (2020) Rapid method for generating designer algal mitochondrial genomes. *Algal Research*, 50, 102014.

Brumwell, S.L., MacLeod M.R., Huang T., **Cochrane R.R.,** Meaney R.S., …
Karas, B.J. (2019) Designer *Sinorhizobium meliloti* strains and multi-functional
vectors enable direct inter-kingdom DNA transfer. *PLoS ONE 14*(6): e0206781.

PRESENTATIONS

The cloning and engineering of diatom mitochondrial genomes in yeast and
bacteria. Molecular Life of Diatoms 6, Virtual Meeting. July, 2021 (Poster/Talk).

The cloning and engineering of diatom mitochondrial genomes in yeast and
bacteria. Phycological Society of America (PSA), Virtual Meeting. July, 2021
(Poster/Talk).

Rapid method for generating designer algal mitochondrial genomes. International
Conference on Engineering Synthetic Cells and Organelles, Virtual Meeting.
May, 2021 (Poster).

Development of methods for the generation and delivery of designer algal
mitochondrial genomes. Graduate Research Symposium, University of Western
Ontario, London, ON, Canada. May, 2020 (Talk).

Rapid for generating designer algal mitochondrial genomes. Build-A-Cell
Workshop #6, Mountain View, CA, USA. January, 2020 (Poster).

Development of whole mitochondrial genome transformation for the introduction
of multiple simultaneous genetic modifications in *Phaeodactylum tricornutum.*
Synthetic Biology Symposium 4.0, Waterloo, ON, Canada. May, 2019
(Poster/Talk).

Development of methods for the generation and delivery of designer algal
mitochondrial genomes. London Health Research Day, London, ON, Canada.
April, 2019 (Poster).

Development of synthetic organelle genomes for commercial and scientific use.
Canadian Algal Workshop, Toronto, ON, Canada. February, 2019 (Poster).

Development of synthetic organelle genomes for commercial and scientific use.
Western Synthetic Biology Symposium 3.0, London, ON, Canada. July, 2018
(Poster).

Development of synthetic organelle genomes for commercial and scientific use.
London Health Research Day, London, ON, Canada. May, 2018 (Poster).

Development of synthetic organelle genomes for commercial and scientific use.
Harold B. Stewart Memorial Lecture and Research Showcase, London, ON,
Canada. January, 2018 (Poster).

Development of synthetic organelle genomes for commercial and scientific use.
Symposium on Synthetic Biology 2.0, London, ON, Canada. July, 2017 (Poster).

TEACHING AND MENTORING EXPERIENCE

Teaching Assistantship, University of Western Ontario

Biochemistry 3386B: Clinical Biochemistry	2020
Biochemistry 3381A: Biological Macromolecules	2019
Biochemistry 2280A: Biochemistry and Molecular Biology	2019
Biochemistry 3381A: Biological Macromolecules	2018
Biology 1002B: Biology for Science II	2018

Undergraduate Mentorship, University of Western Ontario

Anya Zomer	Volunteer	2021 – 2022
Emily Carvalhais	4th year Biochemistry thesis student	2021 – 2022
Samir Hamadache	4th year Biochemistry thesis student	2018 – 2019
Jennifer Davis	3rd year Biochemistry thesis student	2018 – 2019
Daniel Tsyplenkov	4th year Biochemistry thesis student	2017 – 2018
Samuel Tholl	Volunteer	2018 – 2020
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Jiayi Wang	Volunteer	2018 – 2019
SynBio Hacks Case Competition		2018
Air Forman, Angelo Empleo, and Ayushi Bhatt		
Western Synthetic Biology Research Program (WSBR)		2017 – 2019

LEADERSHIP AND EXTRACURRICULAR ACTIVITIES

Canadian Medical Hall of Fame, Brand Ambassador (London, ON),	2022 – Present
National Scholarship Application Committee, Reviewer (UWO),	2022
InterACT, Mentor (London, ON),	2020 – Present
RotarACT, VP of Community Events (London, ON)	2020 – 2021
Forest City SynBio Steering Committee, Member (London, ON),	2019 – 2021
Biochemistry Graduate Student Association, Chair (UWO),	2019 – 2021
Biochemistry Graduate Student Association, Member (UWO),	2018 – 2019
Be-Al-U-Can-B, Volunteer (UWO),	2017 – 2020
Raising Hope, Volunteer (UWO),	2017 – 2020
Retiring with Strong Minds, Volunteer (UWO),	2017 – 2018

RELEVANT WORK EXPERIENCE

Volunteer   January – September 2017
Dr. David Edgell's Lab, University of Western Ontario, London, ON, Canada.

Research Lab Assistant   May – September 2015
Dr. Jeremy McNeil's Lab, University of Western Ontario, London, ON, Canada.