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The development of Sinorhizobium meliloti and Deinococcus radiodurans as chassis for synthetic biology applications

Stephanie L. Brumwell, *The University of Western Ontario*

Supervisor: Karas, Bogumil J., *The University of Western Ontario*
Co-Supervisor: Edgell, David R., *The University of Western Ontario*
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Abstract

Microorganisms can be harnessed for bioproduction and biotechnology to further global efforts in agriculture, health, manufacturing and sustainability. Traditional microbial chassis used for these purposes are the most well-characterized bacteria and yeast, *Escherichia coli* and *Saccharomyces cerevisiae*. Synthetic biology can be used to facilitate engineering of microbial chassis with new or improved traits. However, there is a need to expand the number and diversity of available microbial chassis to include microorganisms that have innate genetic, metabolic and physiological characteristics that we could make use of. Two such bacteria include the nitrogen-fixing plant symbiont, *Sinorhizobium meliloti,* and the polyextremophile, *Deinococcus radiodurans*. While some progress has been made toward the development of these chassis, further strain and tool developments are required to unlock their full potential. Here, I present the expansion of the genetic toolkits for *S. meliloti* and *D. radiodurans* to improve their utility as bacterial chassis for synthetic biology applications.

First, I engineered a genome-reduced strain of *S. meliloti* as a novel conjugative host and demonstrated the transfer of multi-host shuttle vectors to bacteria, yeast and microalgae. Then, I developed a conjugative protocol to transfer DNA from *E. coli* to *D. radiodurans* and showed its utility through the generation of robust systems for conjugation-based genome engineering and whole genome cloning *in vivo*. Using this method, I cloned the large (178 kb) MP1 megaplasmid from *D. radiodurans* in *E. coli*. Finally, I developed a strategy to create seamless gene deletions in *D. radiodurans* which was demonstrated through the sequential genetic knockout of four restriction-modification systems.

The tools and strains developed in my thesis will add to the growing genetic toolkits of *S. meliloti* and *D. radiodurans*. The establishment of *S. meliloti* as an alternative chassis for interkingdom DNA transfer will allow for the study and engineering of agriculturally-relevant microorganisms and modulation of microbial communities. Likewise, the expansion of genetic tools will establish *D. radiodurans* as a microbial platform for industrial applications and the study of extremophile biology. These bacterial chassis will complement the use of traditional microbial chassis, broadening the potential solutions synthetic biology could offer.

Keywords: *Sinorhizobium meliloti, Ensifer meliloti, Deinococcus radiodurans*, synthetic biology, chassis, conjugation, genome engineering, genetic tools, multi-host shuttle plasmids, designer microbes

Lay Summary

Microorganisms like bacteria, yeast and algae can be used to produce food, medicine, building materials and improve the environment. Well-known examples of this include using yeast to make bread or bacteria to make pharmaceutical drugs like insulin. *Sinorhizobium meliloti* and *Deinococcus radiodurans* are two bacteria that naturally have beneficial traits and functions. *S. meliloti* lives in soil and helps improve plant growth, while *D. radiodurans* is able to tolerate harsh conditions such as drought or exposure to radiation. However, much like you would use tools to renovate a house, in order to work with these microorganisms, we need to have tools to make changes to their genetic information. Genetic information (*i.e.*, DNA) is composed of biological building blocks in a particular sequence that determines the traits and functions of all living things. Using synthetic biology, we can engineer organisms by adding, removing or changing these building blocks. The purpose of my thesis is to expand the genetic toolbox of *S. meliloti* and *D. radiodurans* so that they can be engineered more easily.

To achieve this, I demonstrated efficient methods to introduce DNA into *S. meliloti* and *D. radiodurans*, including conjugation. Conjugation is the transfer of DNA directly from one organism to another. Using conjugation, I developed new strategies to alter the genetic information of these organisms through the addition or removal of small or large segments of DNA. Finally, I demonstrated the ability for *S. meliloti* to conjugate DNA directly to bacteria, yeast and algae to allow for easier engineering of these microorganisms.

The genetic tools described in this thesis, coupled with the unique qualities of *S. meliloti* and *D. radiodurans,* make them attractive for research and commercial use. Now, the process to insert DNA encoding a new function, remove unnecessary DNA, or look more closely at DNA to better understand the biology of these bacteria, is simpler and faster. The improved ability to engineer *S. meliloti* and *D. radiodurans* will allow us to accelerate their use in creating solutions to global challenges in agriculture or industry, such as increasing crop growth or removing toxins from nuclear waste sites.

Co-Authorship Statement

For the work presented in Chapter 2, 3 and 4, S.L. Brumwell and B.J. Karas conceived the experiments. S.L. Brumwell performed the experiments and S.L. Brumwell and B.J. Karas analyzed the results. S.L. Brumwell created the figures and wrote the manuscripts with input from B.J. Karas. Exceptions and additional contributions for each Chapter are noted below.

In regards to Chapter 2:

T.C. Charles and T.M. Finan contributed to the conception of experiments. M. MacLeod performed experiments to create the *S. meliloti* Δ*hsdR* strains and pAGE2.0 plasmid stability assay, analyzed results, and created figures for Figure 2-3c (which I modified), and Supplemental Figure B-1. R. Cochrane assisted in conducting and analyzing the results for the 96-well plate conjugation experiments for Figure 2-2e. Under S. Brumwell's supervision as an undergraduate thesis student, T. Huang performed PEG-mediated transformation experiments for Supplemental Figure B-3 and B-4, and helped analyze results. O. Matysiakiewicz performed experiments to generate the *E. coli* Δ*dapA* strain. M. Zamani provided some of the *S. meliloti* strains used. B.J. Karas of Designer Microbes Inc. built the pAGE and pBGE plasmids and provided the gel for Figure B-2. M. MacLeod, T. Huang, T.C. Charles, and T.M. Finan assisted in writing the manuscript.

In regards to Chapter 3:

D.R. Edgell contributed to the conception of experiments. Under S. Brumwell's supervision as an undergraduate intern, K.D. Van Belois assisted in performing experiments for cloning of the MP1 plasmid and contributed multiplex PCR gels for Figure 3-6E and Supplemental Figure C-3. D.J. Giguere performed the Oxford Nanopore sequencing and analysis and created Figure 3-6.

In regards to Chapter 4:

Under S. Brumwell's supervision as an undergraduate thesis student, K.D. Van Belois assisted in performing experiments for the generation of the ΔRM1 to ΔRM1-4 *D. radiodurans* knockout strains and contributed MPX gels for Figure 4-6C and 4-7B.

Dedication

This thesis is dedicated to everyone who guided and steadied me on this journey: my supervisors, lab mates, family and friends.

“The ending isn't any more important than any of the moments leading to it.”

- To The Moon

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations and Symbols

~	approximately
°C	degrees Celsius
α	alpha
µE	microeinstein
µg	microgram
µL	microliter
µM	micromolar
Arg	arginine
ATCC	American Type Culture Collection
BAC	bacterial artificial chromosome
bp	base pair
Cas9	CRISPR-associated protein 9
cat	chloramphenicol acetyltransferase
CFU	colony forming unit
CRISPR	clustered regularly interspaced short palindromic repeats
DAP	diaminopimelic acid
DBTL	design-built-test-learn
DNA	deoxyribonucleic acid
EMS	ethyl methanesulfonate

FDA	Food and Drug Administration (FDA)
Flp	flippase
FRT	flippase recognition target
g	gram
g	gravity
G+C content	guanine and cytosine content
gfp	green fluorescent protein
gusA	β-glucuronidase
h	hour
HF	high fidelity
HIS	histidine
Inc.	incorporated
kb	kilobase
kV	kilovolt
L	liter
lacZ	β-galactosidase
LB	Luria broth
Mb	megabase
MHS	multi-host shuttle
min	minute

mL	milliliter
MPX	multiplex
nat	*N*-acetyltransferase
ng	nanogram
nptII	neomycin phosphotransferase II
Ntc	nourseothricin N-acetyl transferase
OD	optical density
ORF	open reading frame
oriT	origin of transfer
PCR	polymerase chain reaction
PEG	polyethylene glycol
PHA	polyhydroxyalkanoate
PPP	pentose phosphate pathway
R-M	restriction-modification
rfp	red fluorescent protein
RNA	ribonucleic acid
RPM	revolutions per minute
s	second
sddH_2O	sterile double-distilled water
SNP	single nucleotide polymorphism

TALENs	transcription activator-like effector nucleases
TDNA	transfer deoxyribonucleic acid
TE	tris-EDTA
TGY	tryptone-glucose-yeast extract
Ti	tumour inducing
tRNA	transfer ribonucleic acid
UV	ultraviolet
WT	wild-type
w/v	weight/volume
X^R	resistant
X^S	sensitive
YAC	yeast artificial chromosome
YPD	yeast extract-peptone-dextrose
ZFNs	zinc-finger nucleases

Chapter 1

1 Introduction

1.1 Biotechnological potential of microbes

According to the United Nations, the world population is predicted to reach approximately 10 billion by 2050, nearly quadrupling the number of people that were on Earth only a century ago (1,2). With a rising global population, there is an increased demand for resources: food, water, fossil fuels, pharmaceuticals, construction materials, and so on. Many of these supplies are either made from non-renewable materials or take a considerable amount of time to replenish. Microorganisms are a renewable resource that can be industrially scaled quickly and affordably, making them attractive candidates for applications in agriculture, manufacturing, health, and sustainability (3). The recent coronavirus pandemic has emphasized the importance of sustainability in times of resource shortages and highlighted how biotechnology can be used to provide rapid solutions to global challenges.

As of 2020, the McKinsey Global Institute reported that 60% of material economic inputs could be produced biologically (4). Microbes, including bacteria, fungi and algae, possess useful characteristics that can be leveraged for cost-effective bioproduction. Many microbes naturally produce high-value products (*e.g.,* alcohol, antibiotics, pigments, polyunsaturated fatty acids, vitamins) or have enzymatic functions allowing for desirable processes to take place (*e.g.*, fermentation, bioremediation, plastic degradation) (5,6). Some well-known examples include using *Saccharomyces cerevisiae* (*i.e.,* brewer's yeast) in the fermentation of beer or baked goods (7), bacteria and fungi for antibiotic or drug production (8,9), microalgae as feedstock for biofuel production (10), and commensal gut microbes such as *Lactobacillus* as probiotics in dairy products (11).

Microbial bioproduction is a more sustainable approach to manufacturing compared to traditional industrial processes, largely due to the raw materials fed into the industrial process (*i.e.*, feedstocks). Renewable feedstocks such as carbohydrates, one-

carbon compounds, recycled materials or waste can be used to fuel microbial biofactories, whereas traditional chemical feedstocks typically require the consumption of non-renewable materials such as petroleum, coal, and gas (12,13). Bioproduction often takes advantage of the innate enzymatic activity of microbes. Though, synthetic biology tools can be used to fully capitalize on the vast benefits of microbes, engineering them to be more well-suited for a desired function and providing unparalleled opportunities for innovation (14,15).

1.2 Synthetic biology

Synthetic biology is an interdisciplinary field merging the study of living organisms with engineering approaches to create novel or improved biological systems (15). Over the last 20 years, synthetic biology has progressed immensely as a result of the genome revolution. Our ability to read and write genomes has advanced through the decreased cost of DNA sequencing and synthesis and the development of improved tools for genome assembly, engineering, comparative analysis and computational modelling (16,17). These technological advancements have simplified the process of microbial engineering and are now critical components of the design-build-test-learn (DBTL) cycle that synthetic biology is known for (Figure 1-1).

Using synthetic biology, microbes can be engineered or built *de novo* with characterized parts and tools to endow the organism with new or improved functions (15). Synthetic biology can enable the engineering of organisms to enhance the production of native compounds or to produce heterologous or synthetic products that do not exist in nature (13). For example, organisms can be genetically engineered to produce essential nutrients (*e.g.*, carotenoids, vitamins) (18), with biotherapeutic properties (19), or for improved industrial performance by optimizing product yield or increasing their tolerance to harsh conditions like desiccation (20). In addition to industrial applications, synthetic biology can streamline organisms for foundational research, enabling complete control over the genome and providing abundant opportunities for chassis development, genetic explorations and biotechnological applications.

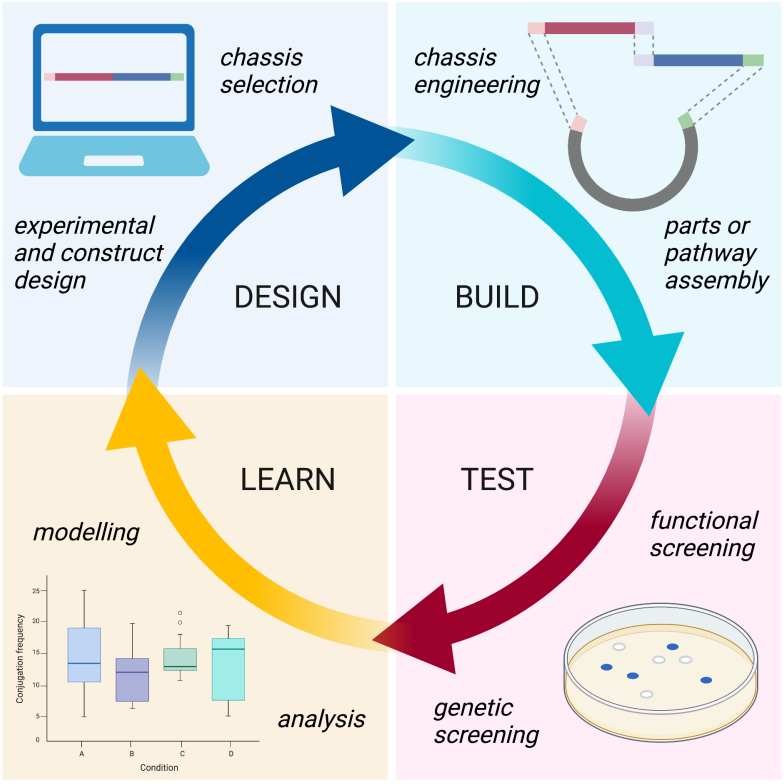

Figure 1-1. Design-build-test-learn (DBTL) cycle. The DBTL cycle can be applied to any synthetic biology project or hypothesis. Design: *in silico* tools are used for the design of DNA constructs and implementation into experimental design with consideration for the optimal chassis organism based on the intended application. Build: DNA constructs are built through assembly or synthesized *de novo* and/or chassis strains are engineered. Test: genetic or functional screening is performed, often utilizing high-throughput technologies. Learn: computational analysis and modelling of data informs the generation of novel hypotheses, allowing the cycle to begin again. Created with BioRender.com.

1.3 Microbial chassis

In the manufacturing of cars, a chassis is the rudimentary support framework that all other components of the vehicle are built upon. While the basics of automotive chassis are roughly the same, they can vary in size and shape depending on the vehicle or manufacturer (21). The word chassis bears an analogous meaning in synthetic biology, where a microbial chassis refers to a living organism that acts as the framework to build or express genetic pathways, and is equipped with the resources and tools for their engineering or expression (22). Much like we would not use the exact same framework to manufacture a truck and a car, the choice of microbial chassis can differ widely based on the intended industrial or academic application and differences in their innate biological characteristics.

1.3.1 Traditional chassis organisms

Escherichia coli and *S. cerevisiae* are the most commonly used bacterial and eukaryotic chassis, respectively, for both bioproduction as well as foundational research (23,24). These model organisms have been studied extensively for the last century, leading to their well-characterized physiology, ease of handling in the laboratory, and extensive genetic toolkits of genetic parts (*e.g.*, promoters, terminators, selective markers), plasmids, and robust protocols for manipulation. These parts and plasmids are often readily available in repositories like the Registry of Standardized Biological Parts (http://parts.igem.org/), the *Saccharomyces* Genome Database (https://www.yeastgenome.org/) and Addgene (https://www.addgene.org/), making them easily accessible for use (25).

Since being sequenced in 1997 (26), the *E. coli* genome has been characterized through metabolic modelling, the generation of gene knock-out libraries (*e.g.*, Keio collection), and the engineering of reduced or synthesized genome strains (27–30). *E. coli* can be easily engineered using robust methods for genetic transformation, *in vivo* DNA assembly and a multitude of genome editing technologies. For the aforementioned reasons, *E. coli* is often the first choice for a bacterial chassis and is also commonly used

as an intermediate chassis for protein expression as well as reliable plasmid induction, isolation and sequencing (31). This is made possible through the availability of specialized strains and plasmids for cloning and expression with features such as variable copy number and stability. *E. coli* has minimal nutritional requirements for growth and a short generation time (~20 minutes) that expedites experimental turnover which can permit rapid scale-up for bioproduction (32). The use of *E. coli* for bioproduction can be traced back to the 1960's, where it was engineered to produce the amino acid L-threonine, and later in the 1980's for the production of human insulin through single gene mutations and insertions. Since then, *E. coli* has been used as a chassis for a plethora of bioproduction applications (33,34). Moreover, *E. coli* aids in the engineering of other model and non-model organisms by acting as an efficient conjugative donor for DNA delivery, which has been demonstrated to diverse cell types including bacteria (Gram-negative and Gram-positive), yeast, microalgae, and mammalian cells (35–38).

Many of the characteristics that make *E. coli* a popular chassis are also exhibited by *S. cerevisiae.* As the first eukaryotic organism to be fully sequenced in 1996, the genome of *S. cerevisiae* is well-characterized (39). Engineering eukaryotic genomes can introduce additional challenges, however *S. cerevisiae* is a diploid eukaryote that can be cultured as a haploid cell, improving the ease of genetic manipulation (40). Engineering efforts in this chassis have resulted in a complete library of single gene deletion mutants and strains of yeast containing both reduced and synthetic genomes (*e.g.*, Sc2.0, Sc3.0) (41–44). *S. cerevisiae* is commonly harnessed for homologous recombination-based *in vivo* DNA assembly (45), which has notably been used in the creation of whole bacterial genomes (46,47). *S. cerevisiae* is also often used to study models of human disease (*e.g.*, cancer, metabolic disorders), as roughly a quarter of yeast genes have human homologs (48). Unlike *E. coli* and several other attractive microbial chassis, *S. cerevisiae* has been given "generally regarded as safe" status by the Food and Drug Administration (FDA), allowing for its use in the development of products for human consumption. The use of *S. cerevisiae* as a chassis for biotechnology first occurred in the 1990's for the manufacturing of the sugar alcohol xylitol (49); however, it could be argued that the use of *S. cerevisiae* for human benefit was initiated between two thousand to two million years ago, according to the first record of fermented bread (50).

1.3.2 The need for alternative chassis

There is a need to increase the number and variety of available chassis beyond *E. coli* and *S. cerevisiae*. These traditional chassis organisms are commonly adapted in biotechnology because their biology has been extensively studied, they have vast genetic toolkits, and are amenable to genomic manipulation. However, there are some genetic engineering and physiological limitations of *E. coli* and *S. cerevisiae* that makes them unsuitable platforms for every desired application. Both chassis are limited by their inability to assemble or propagate genetic information that is large (>100 kb) and GC-rich (>40%) without additional engineering (51). Complications can arise when these microbes are engineered with exogenous genes or long biosynthetic pathways, leading to the expression of products that are toxic to the host or burdening the host's metabolic system (51–53). These limitations narrow DNA engineering possibilities and strategies. Therefore, the GC-content and size of desired DNA constructs should be considered during chassis selection. With this in mind, establishment of a suite of chassis with genomes of varying GC-contents would be beneficial.

With advances in DNA reading and writing, the main bottleneck of whole genome synthesis or engineering remains delivery to desired hosts (17). Despite *S. cerevisiae* being used extensively as a host for building synthetic genomes, the current method for delivering genomes, called genome transplantation, has only been demonstrated successfully a handful of times to *Mycoplasma* and *Mesoplasma* species (46,47,54). Characteristic of the Mollicute class, these species have small genomes, an alternate genetic code, and lack a cell wall allowing for ease of synthesis, cloning and delivery of the genome into the recipient cell (46). This strategy may not be possible with more complex recipient organisms; therefore, it is necessary to develop high-efficiency methods for delivering large-scale DNA within and between a wide range of chassis (17). Conjugation is a direct method of DNA transfer that is common in bacteria and could provide a solution, however *S. cerevisiae* is unable to transfer DNA using this method. Accordingly, there is a need to develop chassis organisms with the capacity for

transferring large DNA constructs or genomes by interspecies and/or interkingdom conjugation (40).

Although traditional chassis are frequented for bioproduction, they are mesophilic, have low tolerance to toxic compounds and other stressors, use a limited variety of carbon sources (namely glucose), and have metabolic constrictions that can result in diminished production efficiency (23,24,55). As such, *E. coli* and *S. cerevisiae* may not be the ideal chassis for applications in extreme or niche environments. To ensure that synthetic biology research can be translated into practical, real-world innovations, there is a need to identify more diverse chassis that can thrive in a range of environments. It is particularly important to consider whether the chassis of interest can withstand the conditions of the final destination, which can significantly differ from laboratory conditions and may include stressors such as extreme temperatures, salt concentrations, pH levels, desiccation, and ethanol concentrations (56,57). Non-traditional organisms may innately possess attractive physiological characteristics that could be useful for applications in biotechnology.

1.3.3 International efforts for chassis expansion

The importance of chassis selection for synthetic biology research and applications has begun to emanate throughout the field. Although the number of published manuscripts in the field of synthetic biology has vastly increased in the last 20 years, it is only in the past 10 years that alternative chassis development has been prioritized (Figure 1-2). There have been several collaborative international efforts that are dedicated to the generation of more diverse microbial chassis. These include projects aimed at improving technology and decreasing the cost of DNA sequencing and synthesis, as well as characterizing non-model chassis organisms and their genetic parts. Some examples of these efforts, past and present, include the Human Genome Project (1990) (58), Minimum Genome Factory (2001) (28), Genomes to Life (2002) (16), “973” - Design and Construction of Microbial Consortia (2014) (59), and Human Genome Project – Write (2016) (60). Aside from these scientific research and development consortia, several biotechnology companies are

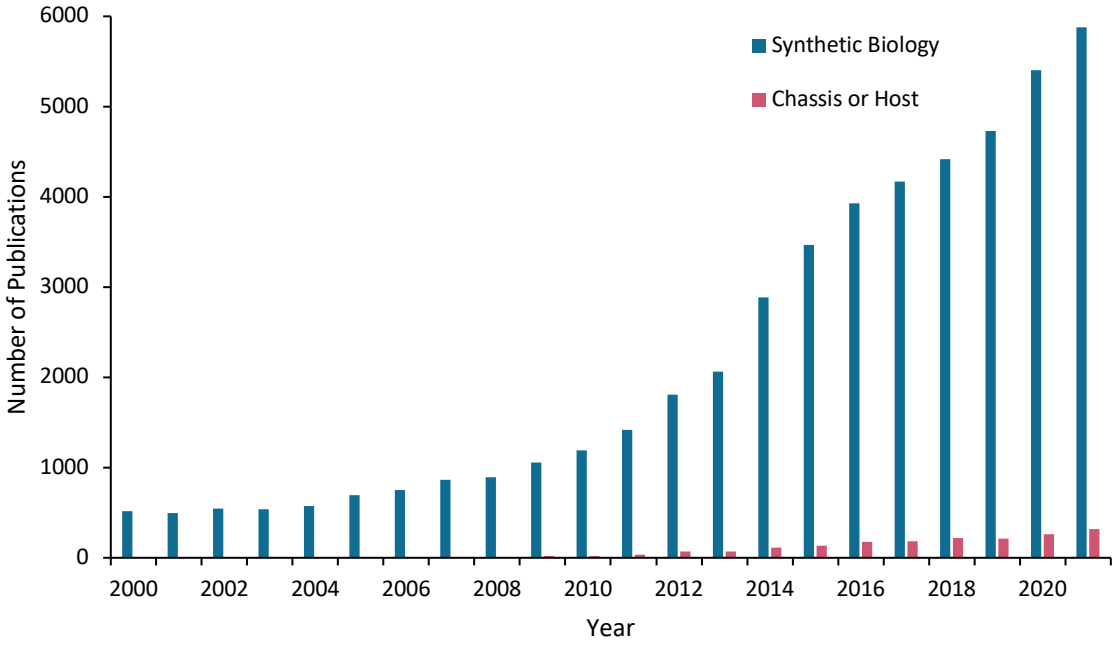

Figure 1-2. Intersection between synthetic biology and chassis research. Graphical representation of the number of PubMed search results for publications using keywords "synthetic biology" (blue) and "synthetic biology" + "chassis" or "host" (pink) from 2000 to 2021 (source: PubMed, accessed in May 2022).

also dedicating time and resources to the development of non-model chassis including Cultivarium, Ginkgo Bioworks, and Pivot Bio, to name a few. Of these, Cultivarium is specifically seeking to create automated systems for the development of "wild microbes" as chassis for applications in biotechnology (https://www.cultivarium.org/).

1.4 Chassis characteristics

The great diversity of existing microbes offers an untapped resource of unique genetic, metabolic and physiological characteristics that could be exploited by synthetic biologists in the development of new microbial chassis. Ideal characteristics of a chassis organism often include i) a simplified, characterized genome, ii) a collection of genetic tools and reliable DNA delivery methods, iii) simple or unique metabolic requirements, and iv) physiological tolerance to the environmental conditions of the intended application (Figure 1-3) (22,61).

1.4.1 Simplified genome

Microorganisms with sequenced and well-annotated genomes are ideal for use as chassis as this genetic information is imperative for characterization of genetic parts and targeted genome engineering. Chassis with simplified genomes are particularly useful for bioproduction and biotechnology because a simplified genetic background can reduce undesirable regulatory interactions, decrease the expression of non-essential products, and allow for the diversion of cellular resources towards a desired process (62). For example, reducing the genome size of *E. coli* by 22% resulted in increased cell density and increased L-threonine yield by 2-fold (28). Likewise, reducing the *S. cerevisiae* genome by just 5% led to increased yield of ethanol by approximately 2-fold (44). Some microbes of interest naturally have small genome sizes (*e.g.*, *Mycoplasma genitalium* <600 kb) (63); however, the genome size of most bacteria are in the megabase range and could benefit from genome simplification (64). Simplified genomes can be developed by either top-down genome reduction or bottom-up genome synthesis.

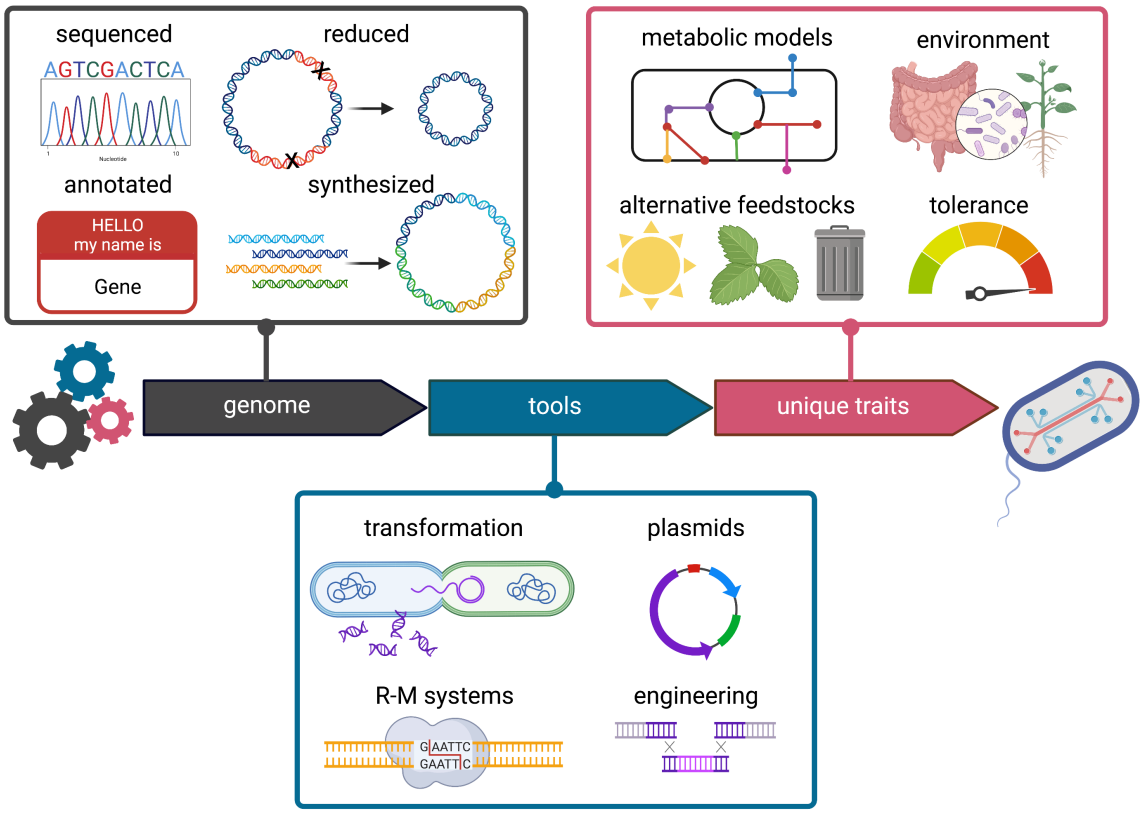

Figure 1-3. Chassis characteristics. An ideal chassis will have a sequenced, annotated and simplified genome (through genome reduction and/or synthesis). It will also have a suite of available genetic tools and methods for introducing DNA. Finally, an alternative chassis will possess unique traits beneficial for the intended applications; for instance, utilization of renewable carbon sources, native to the environment of the final destination (*e.g.*, gut microbiome, plant rhizosphere), or tolerance to industrial or stressful conditions. Created with BioRender.com.

Top-down genome reduction involves identifying and removing redundant or dispensable sequences in the genome while retaining essential sequences required for life or a desired function (*e.g.*, symbiosis). Dispensable sequences could include transposable elements, phage remnants, non-functional genes, or genes required for niche-specific metabolism (29). This strategy is often aided by the use of large-scale comparative genomics or *in silico* methods like MinGenome (62) and is followed by empirical validation. One of the first examples of genome reduction was performed by Kolisnychenko et al. (2002), using comparative genomics to identify regions of the *E. coli* MG1655 genome that were not present in other *E. coli* strains (29). Deletion of twelve strain-specific genomic islands in *E. coli* MG1655, presumably obtained through horizontal gene transfer, resulted in an 8.1% reduction in genome size and little to no physiological effects (29).

Genome synthesis is a bottom-up approach that involves building a microbial genome *de novo* and installing it in a chassis organism. This strategy is made possible due to the decreasing cost of DNA synthesis and improved DNA manipulation techniques as previously mentioned. This has been achieved by synthesizing and assembling short, overlapping DNA fragments (*e.g.*, 1 kb) via homologous recombination in *S. cerevisiae* (61). Following assembly, the synthetic genome must be tested for functionality or viability, which has been performed using genome transplantation. This method was implemented by researchers at the J. Craig Venter Institute to create the first fully synthetic genome (*i.e.*, *JCVI-syn1.0*) originating from *Mycoplasma mycoides* (65). The synthetic genome was 11% smaller than the wild type, with a total size of 1079 kb. Further minimization of this genome by 56% at 531 kb was accomplished to produce *JCVI-syn3.0* (66). Strategies for genome synthesis have also been investigated and proposed in eukaryotic organisms including *S. cerevisiae* (*Sc2.0* and *Sc3.0*) (42,43) and the diatom microalgal species *Phaeodactylum tricornutum* (*Pt-syn1.0*) (67). Rather than fully assembling the genome prior to delivery, sections of the native chromosome can be replaced one-by-one with synthetic parts until the entire chromosome is replaced. This strategy was used in the Synthetic Yeast Genome Project towards the goal of synthesizing the first eukaryotic genome (*i.e.*, *Sc2.0*) (42).

Both described strategies for simplifying genomes have been shown to be successful, though consideration of their advantages or disadvantages is imperative based on the genome of interest. The advantage of using a top-down method is that the genome originates from a fully functional, living cell. By iterative removal of DNA, reduction can be halted once bioproduction goals have been reached or can be backtracked if the cell becomes nonviable or an undesirable phenotype is introduced (62). The bottom-up method for constructing synthetic microbial chassis is appealing to researchers because it allows for rational design of genomes allowing for the integration of genes or biosynthetic pathways for a desired application, genome recoding, and genome minimization. However, "booting up" a genome is a complex process since it often involves the transfer of very large DNA constructs (68). Regardless of the approach, a simplified genome can be used as a more predictable chassis for biotechnology and to help researchers understand the fundamental requirements for life.

1.4.2 Genetics tools

A basic genetic toolkit is fundamental for engineering any new chassis organism. Toolkits should contain a selection of available genetic parts and plasmids, as well as methods for delivering and manipulating DNA to enable strain engineering (22). Genetic parts for efficient gene expression are necessary to produce a protein of interest in a host organism. Most of these parts are regulatory elements like promoters and terminators. Since transcriptional machinery differs between organisms, these regulatory elements often only function in closely related species, and therefore must be identified or developed on a species-to-species basis. Promoters are necessary for driving gene expression and can be constitutively active or regulated by transcription factors (*e.g.*, inducible, tissue-specific), whereas terminators signify the end point of transcription. Both of these elements can vary in strength or efficiency and are often taken from ubiquitously expressed genes endogenous to the chassis, though it is also possible to design and synthesize them *de novo* (69,70). Having a collection of these genetic elements for a chassis is important to tailor gene expression to the desired level based on the application.

Incorporating elements for stable maintenance and selection of the introduced DNA in the chassis is the next obstacle. Transgenes can be introduced into an organism as linear DNA constructs or on a plasmid that can integrate into the host genome or replicate autonomously (*i.e.*, an episome). For stable plasmid maintenance, origins of replication (and centromeres for eukaryotic hosts) are required and can be identified from chromosomes or plasmids native to the chassis (71,72). While some origins of replication can be used amongst a variety of host organisms, known as broad-host-range origins (73), there is often a need for species-specific origins of replication in non-model organisms. Other considerations for plasmid design include plasmid segregation, copy number, and size limitations (13). Whether the DNA is destined for genome integration or maintained as an episome, a method for selecting cells that have taken up the DNA is required. Common selective strategies include the use of antibiotics or nutritional deprivation (*i.e.,* auxotrophs) where the introduced DNA contains a gene conferring resistance to the antibiotic or a missing essential nutrient, respectively. Alternatively, reporter genes can be incorporated for the ease of visually screening transformants, such as green fluorescent protein (*gfp*) or β-galactosidase (*lacZ*). A final consideration is the inclusion of any genetic parts required for the intended plasmid delivery method, such as an origin of transfer (*oriT*) for conjugation.

Once all of these genetic elements are in place, a crucial step in microbial engineering is delivering the genetic construct into the organism, bypassing the cell wall and/or cell membrane. Some species are naturally competent, meaning that they have the ability to uptake DNA from the environment (74), but for those that are not, there are a few traditional methods that have been developed to deliver DNA to a wide range of species including chemical transformation (75,76), electroporation (77), and conjugation (35). Once inside the cell, the foreign DNA must evade native restriction-modification (R-M) systems that recognize and degrade differentially methylated DNA (78,79). Thus, it is important to analyze the methylome of candidate chassis organisms to identify and potentially remove these R-M systems. The choice of transformation method can influence the extent of DNA degradation by the host cell. Since many restriction endonucleases cleave double-stranded DNA, the use of natural transformation (if

competent) or conjugation can help with R-M evasion as both methods can introduce single-stranded DNA into the recipient cell (80).

The ability to carry out gene editing or make genomic modifications is vital to engineer a chassis of interest for industrial or academic applications. Strategies for genome engineering can either be random or targeted. Random mutagenesis can be carried out using DNA-damaging agents such as radiation or chemical mutagens (*e.g.*, ethyl methanesulfonate [EMS]) (81), to create point mutations, or transposons (*e.g.*, Tn5) (82), for random integration of a DNA construct. These methods are useful for generating a library of mutants that can be screened for variances in viability or a desired phenotype, or elucidate genetic mechanisms of interest. For targeted modification of the genome, homologous recombination, recombineering, or RNA-guided nucleases can be used (83). These methods are useful for making precise modifications such as a single nucleotide change or inserting/deleting a gene of interest, while keeping the rest of the genome unaltered. Homologous recombination is easily adapted as a tool for targeted engineering by flanking a construct of interest with sequences homologous to the genomic target (84). Recombineering strategies using lambda red recombination or site-specific recombinases such as the Flp/FRT, Cre/LoxP or *attP-attB* systems can also be used to integrate DNA into the genome. Finally, recent advancements in synthetic biology have allowed for site-specific genome editing using techniques like clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) proteins, transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs) (83).

1.4.3 Metabolic properties and stress tolerance

The metabolic activity of microbes is imperative for sustainable human existence. Microbes facilitate our everyday activities through the preservation and processing of food, breakdown of nutrients in our gut, conversion of organic matter in soil to promote plant growth, and so on. In the development of non-conventional microbial chassis, metabolic modelling or profiles can provide insights into energy utilization and production for optimal cellular functioning (80). The carbon source for industrial

bioproduction accounts for 60% of total production cost (85). As such, developing an array of chassis microbes that utilize different carbon sources, have minimal nutritional requirements and/or can utilize simple substrates could reduce heavy consumption of conventional feedstocks (80,86). Microbial bioproduction has traditionally relied on feedstocks like starch and sucrose, but there is concern that these may not be sustainable sources as the global population increases alongside the demand for food (87). As a result, there has been an increased interest in microbes that can use alternative feedstocks that are more sustainable and inexpensive including organic waste, lignocellulosic biomass (88), sunlight (89), or even recycled materials like plastic (90).

Industrial bioproduction and certain other applications can expose microbial chassis to a number of stressors as a result of the industrial process, environmental conditions, or by-products of feedstock metabolism. These conditions may include desiccation, high temperature, acidic or alkaline pH, osmotic stress, and high ethanol concentration (56,91). The pre-treatment and breakdown of certain feedstocks, like lignocellulose, can lead to the production of inhibitors like weak acids, phenolic compounds and furans that can impact microbial growth (92). Stressful environmental conditions can result in decreased production efficiency as microbes must divert their cellular energy from bioproduction to maintaining homeostasis. Mitigating environmental stressors can be costly as it may require cooling or separation of products or by-products during the industrial process (91,93). Therefore, microbial chassis chosen for industry or other demanding applications would benefit from being robust and stress tolerant. Research is being conducted to endow traditional chassis with these capabilities; however, stress tolerance is often polygenic (*i.e.,* involving several genes) and therefore may be difficult to fully understand or engineer into model organisms (93). Leveraging non-model microbes with innate stress tolerance may prove to be more suitable chassis for industrial bioproduction or applications involving harsh or niche environments as many naturally have stress tolerances exceeding that of *E. coli* and *S. cerevisiae* (93).

Two groups of microbes that are of interest for niche applications include plant symbionts and extremophiles. Rhizobia are agriculturally-valuable bacteria that can be used to promote crop growth as nitrogen-fixing symbionts and native members of the

rhizosphere (94). *Deinococcus* species are extremophilic bacteria that have high stress tolerance, cellular and enzymatic stability; as such, they can be used for applications such as bioremediation of toxic compounds like nuclear waste (95). Thus, two bacteria that are attractive candidates for chassis development are the legume symbiont, *Sinorhizobium meliloti* and the polyextremophile, *Deinococcus radiodurans*. However, one of the main challenges in developing new chassis organisms is the lack of tools for genetic characterization and engineering. Therefore, exploitation of these physical attributes will require directed efforts towards genetic tool development to enable engineering of these bacterial chassis.

1.5 *Sinorhizobium meliloti*

Sinorhizobium meliloti (recently reclassified as *Ensifer meliloti*) is a Gram-negative α-proteobacterium well-known for its symbiotic relationship with legume plants, including agriculturally important alfalfa (*i.e.*, *Medicago sativa*). *S. meliloti* fixes atmospheric nitrogen for the legume, augmenting plant growth, and receives nutrients in return (96). It is easily grown in the laboratory with minimal nutritional requirements, has a slow bacterial growth rate (~two hours) (97) close to that of *S. cerevisiae*, and is non-pathogenic (Biosafety Level 1). As a soil-dwelling microbe, it inhabits a niche environment and has natural interactions with the rhizosphere microbiome and plants. As such, *S. meliloti* is an attractive candidate chassis for synthetic biology applications seeking to improve agricultural practices. Progress has been made toward the development of reduced-genome strains, genetic tools and metabolic models; however, additional tool development is required to take full advantage of the features of this chassis.

To further develop *S. meliloti* as a bacterial chassis, there is a need to expand the utility of genome-reduced strains and the number and complexity of available genetic tools (Figure 1-4). This includes the generation of multi-host shuttle plasmids for the cloning of large-scale DNA and potentially whole chromosomes. In order to transfer

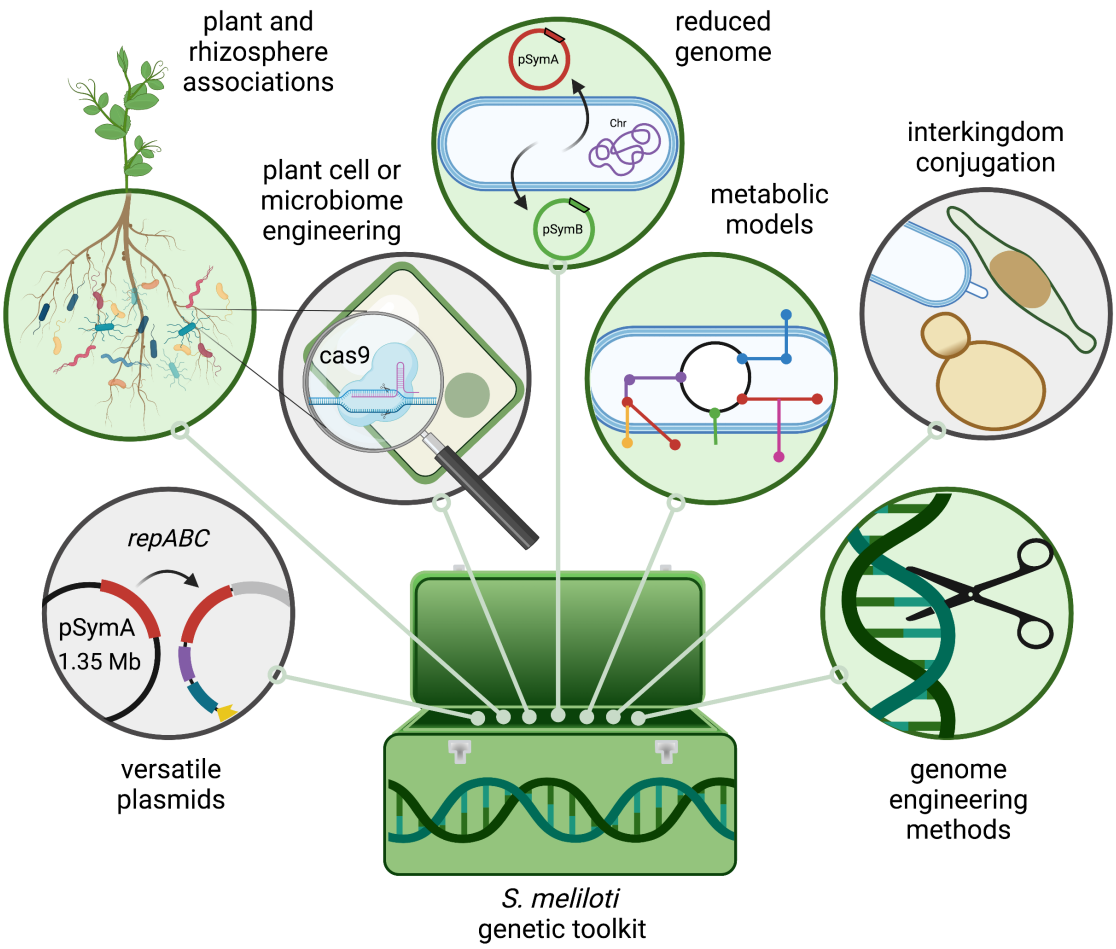

Figure 1-4. *S. meliloti* genetic toolkit. Genetic tools and characteristics of *S. meliloti* that are advanced (green) or need to be developed further (grey). Created with BioRender.com.

large DNA constructs, there is a need to develop high-efficiency methods for DNA delivery to and from *S. meliloti* to a wide range of chassis (17). These tools could enable large-scale DNA delivery and engineering strategies, potentially providing a platform for plant engineering or improvement in the future.

1.5.1 Genome

S. meliloti has a multipartite genome including a chromosome (3.65 Mb) and two large extrachromosomal replicons: the pSymA megaplasmid (1.35 Mb), and the pSymB chromid (1.68 Mb), with an average G+C content of 62% (96). Megaplasmids often encode niche-specific traits and more closely resemble a plasmid, while chromids usually contain essential housekeeping genes and more closely resemble a chromosome (98). Genome reduction experiments have been performed, resulting in derivative strains of *S. meliloti* lacking either or both of the pSymA and pSymB replicons, resulting in an up to 45% reduction of the genome (97). Analysis of pSymB by diCenzo et al. (2013) found that *tRNAarg* and *engA* genes were essential for free-living growth and were therefore moved to the main chromosome in these reduced-genome strains (99).

1.5.2 Genetic tools

In addition to a sequenced, annotated genome (96), some genetic tools have been developed for *S. meliloti*. Stable cloning and shuttle plasmids are available for use, however plasmid diversity is still underdeveloped in this species. *S. meliloti* origins of replication have been identified including *oriC* (71) and the *repABC*-type origins of the native pSymA and pSymB replicons (72). Since these secondary replicons are greater than 1 Mb in size, their origins of replication could be ideal candidates to incorporate onto plasmids as they are capable of maintaining DNA at low copy and large size. Other necessary components for synthetic biology applications have been established including promoters and terminators such as taurine, IPTG and succinate inducible promoters (100–102). Selective markers have been shown to provide resistance to several antibiotics in *S.*

meliloti including gentamicin, spectinomycin, and kanamycin, to name a few, and reporter genes including *gfp*, red fluorescent protein (*rfp)*, *lacZ* and β-glucuronidase (*gusA*) have also been used (103).

In order to engineer *S. meliloti*, tools and methods for DNA uptake and genome manipulation are required. Methods for introducing DNA into *S. meliloti* include freeze-thaw chemical transformation (104), electroporation (105) and conjugation, which often makes use of triparental mating strategies (71). To improve transformation efficiency, researchers have identified and deleted R-M systems in the model *S. meliloti* strain Rm1021 including the *hsdR* gene and the *hsdMSR* operon (105,106).

Strategies for random and targeted genetic engineering in *S. meliloti* have been demonstrated that will further metabolic engineering efforts in this species. Random mutagenesis using Tn5 has been used to elucidate genetic loci involved in a particular phenotype or genetic pathway (107,108). Meanwhile, recombinase-based targeted engineering systems have been implemented such as Cre/*lox*, lambda integrase recombination (*attB-attP*), and Flp recombinase systems (109) in *S. meliloti* with demonstrated uses in deleting genes or large genomic regions (106,109,110). Most recently, targeted genome editing was demonstrated using a CRISPR/Cas9-based system (111). Researchers fused a nicking variant of the Cas9 nuclease to deaminases and demonstrated successful base editing resulting in adenine-to-guanine, cytidine-to-thymine, or cytidine-to-guanine changes. Altogether, there are several demonstrations of random and targeted genome engineering that have made *S. meliloti* more amenable to small- and large-scale manipulation and highlight the potential for *S. meliloti* as a chassis.

1.5.3 Metabolic properties and stress tolerance

The metabolism of *S. meliloti* changes in different environments, whether it is free living in the soil or as a bacteroid in the root nodule of a legume (112). As such, information on the complete cellular metabolism of *S. meliloti* is important in the consideration of experimental design based on the desired application. Metabolomics, metabolic

modelling and reconstruction have been performed for the bacterium in a free living state as well as in a symbiotic state (112–114). In addition, removal of the secondary replicons of *S. meliloti* as previously described has provided novel insights into their metabolic and niche-specific contributions (112). These metabolic models are a useful engineering tool for *S. meliloti* allowing for simulated cellular metabolism as a result of proposed genetic changes.

S. meliloti naturally inhabits soil environments including bulk soil and the rhizosphere (*i.e.*, the area of soil surrounding the plant root system), as well as root nodules of legumes during symbiosis. In the process of symbiosis, *S. meliloti* endures reactive oxygen species released from plant defence systems upon infection as well as the microaerobic environment of the root nodule (115,116). Therefore, this bacterium has adapted to withstand the stressors from these different environments including nutrient, water and oxygen fluctuations and limitations, as well as oxidative and acid stress (116–120). Commercial strains of *S. meliloti* used as seed inoculum must undergo a drying process. The molecular mechanisms driving their ability to withstand desiccation, including DNA repair and accumulation of trehalose stores (121) could make *S. meliloti* a promising chassis for industrial applications where exposure to these stressors are recurrent.

1.5.4 Current and future applications

All of these factors accumulate to make *S. meliloti* particularly appealing for many applications, the most common of which are agricultural and seek to take advantage of the nitrogen-fixing capabilities of this organism. Symbiotic nitrogen fixation plays a vital role in the development of sustainable agriculture, therefore creating improved inoculants of *S. meliloti* to enhance crop growth is of great interest to the scientific and agricultural communities (122). Improvements could come in the form of engineered strains that can outcompete native species in the rhizosphere, express plant growth promoting genes (*i.e.,* auxins), or have tolerance to drought, salt, or other stress conditions present within the soil or industrial processes.

Research towards engineering symbiotic nitrogen fixation between *S. meliloti* and valuable non-legume crops, such as cereals, is also being investigated (123,124). Nitrogen fixation in non-legume crops could potentially be engineered through the development of synthetic symbiosis; however, the development of novel interkingdom interactions can be difficult as hormone signalling and genetic determinants of the plant and bacteria dictate symbiotic specificity (123). Alternatively, plants can be engineered for nitrogen-fixation directly through the integration of nitrogenase into endogenous organelles, namely mitochondria or chloroplasts, as they could provide the oxygen-limitation that this enzyme requires (124). The development of *S. meliloti* as a eukaryotic endosymbiont, where it would serve as a stable nitrogen-fixing organelle within the plant cell, has also been proposed (125). Coupled with the new-found knowledge of the minimal set of genes required for nitrogen-fixation (126), the development of a nitrogen-fixing organelle could be simplified.

As a native member of the rhizosphere with direct associations with valuable crops and other soil-dwelling bacteria, *S. meliloti* has potential for conjugation-based microbiome modulation and for delivery of growth-promoting traits to plants (127). Additionally, *S. meliloti* can be engineered as biosensors for the detection of specific molecules that are indicative of the physiological condition of soil or the composition of the rhizosphere (128). Evidently, the use of *S. meliloti* as a chassis for synthetic biology has many potential agricultural applications, with even more on the horizon of development.

In addition to terrestrial benefits, researchers are starting to think of off-planet agricultural applications (*i.e.,* "astroagriculture") for *S. meliloti*, due to the decrease in arable land on Earth. Harris et al. (2021) investigated the potential for *S. meliloti* to increase the fertility of Martian regolith and found that clover plants grown in simulated Martian regolith had increased biomass when inoculated with *S. meliloti* compared to uninoculated clover at 0.29 g and 0.01 g, respectively (129). The same year, a similar study was performed using the legume *Medicago truncatula* in simulated Martian regolith revealing that nodule formation and genetic markers for nitrogen fixation were present in inoculated samples (130). Beyond agriculture, *S*. *meliloti* could be used as cell

factories for the production of biomaterials and vitamins. For instance, the production of the biodegradable polymer polyhydroxyalkanoate (PHA) has been observed in *S. meliloti* isolates utilizing lactose and whey permeate as a carbon source which are by-products of the butter or cheese-making process (131). In addition, strains of this bacterium have been identified for the production of vitamin B12 (132). Identification of the strains and pathways lays the foundation for further engineering of improved strains and eventually, commercial production of these types of resources.

1.6 *Deinococcus radiodurans*

Deinococcus radiodurans is a well-known extremophile that was first discovered in 1956 inside a can of irradiated meat (133). In fact, it is so well-known for its extreme abilities that it was listed in the Guinness Book of World Records as the "most radiation-resistant lifeform" in 1998 and the "first bacteria proved to survive in space" just two years ago (134). *D. radiodurans* is a Gram-positive, non-sporulating bacterium that forms tetrads during growth, is easily grown in the laboratory with minimal nutritional requirements, has a slow bacterial growth rate (~130 minutes), and is non-pathogenic (Biosafety Level 1) (135). Due to its unique physiological and genetic capabilities, *D. radiodurans* is an attractive candidate chassis for synthetic biology applications; however, for the last 60 years, much of the research into this species has focused on its extremophilic qualities rather than the potential is has as a chassis. As such, little progress has been made towards its development as a chassis, but some research has been performed for characterization of the genome and development of genetic tools.

Ostrov et al. (2019) recently noted that the ability to perform DNA assembly to the extent that can be achieved in *S. cerevisiae* has not yet been demonstrated in any other organism (17). Due to some of the aforementioned limitations for yeast assembly of high G+C content DNA, there is a need to expand the available chassis capable of efficient homologous recombination-based assembly. *D. radiodurans* has the potential to be one such chassis; however, to further its development for synthetic biology applications, the genetic toolkit needs to be expanded (Figure 1-5).

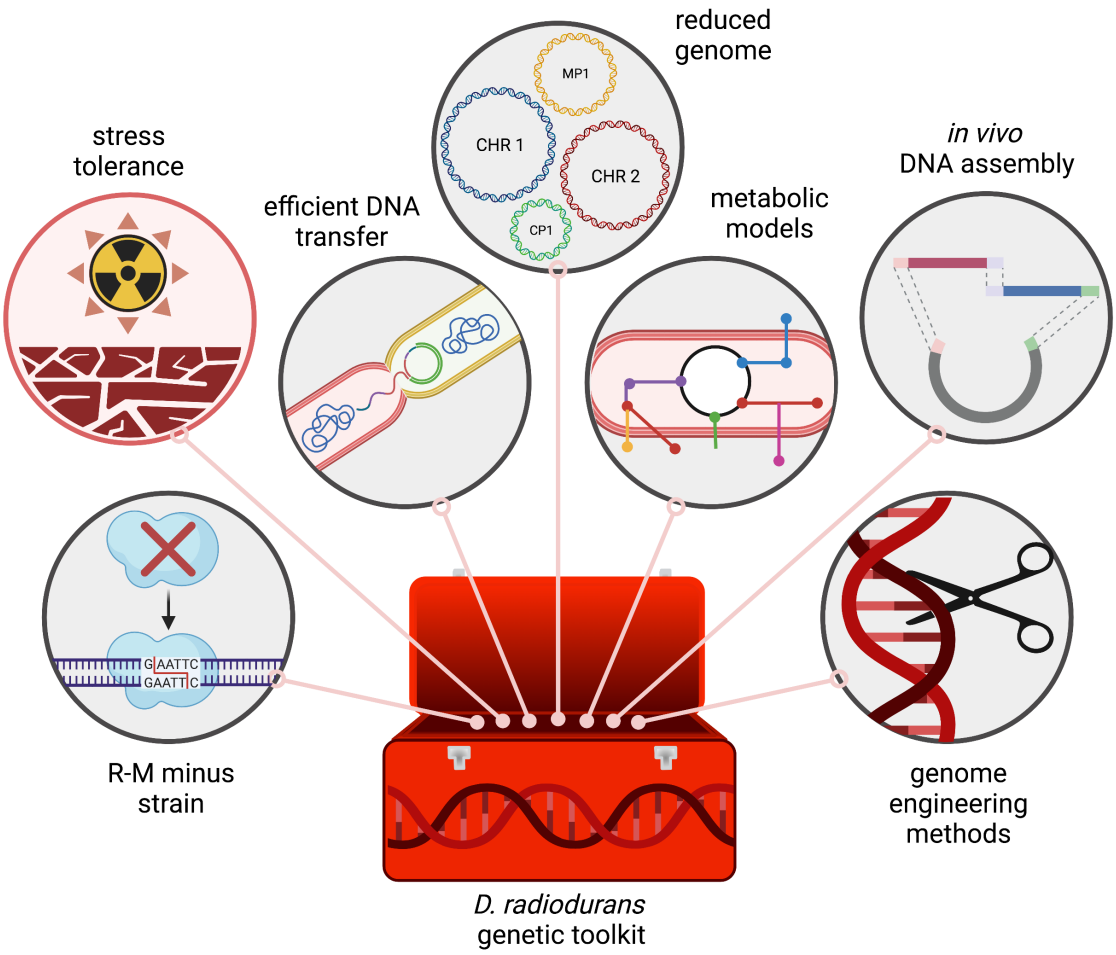

Figure 1-5. *D. radiodurans* genetic toolkit. *D. radiodurans* genetic toolkit. Genetic tools and characteristics of *D. radiodurans* that are advanced (red) or need to be developed further (grey). Created with BioRender.com.

Improved transformation efficiency will be necessary and could be attained through the development of improved DNA delivery methods or through the removal of R-M systems that are currently limiting the uptake of foreign DNA. If DNA could be introduced into this chassis more readily it could be used for the assembly and maintenance of DNA, much like *S. cerevisiae* is, with particular implications for high G+C content DNA.

1.6.1 Genome

Similarly to *S. meliloti*, *D. radiodurans* has a high G+C content (67%) and multipartite genome. Its genome was first sequenced in 1999 by White et al., showing that it is composed of two chromosomes (chromosome 1 and 2) and two large plasmids (MP1, CP1) (136). Comparative genome analysis has been performed and determined that 10-15% of the *D. radiodurans* genome was obtained as a result of horizontal gene transfer (HGT), centralized on the MP1 megaplasmid (137). This organism has a multicopy genome, containing four copies in stationary phase and up to 10 copies in exponential phase (138). This was previously thought to be rare and unique to a select few bacteria; however, it has been observed in other prokaryotes as well including cyanobacteria (139,140).

1.6.2 Genetic tools

A rudimentary genetic toolkit currently exists for *D. radiodurans* with some available genetic parts, plasmids and shuttle plasmids for this species; however it has been noted by Gerber et al. (2015) that the generation of smaller, more versatile shuttle plasmids with a broader host range would further genetic engineering efforts (141–144). Selective markers have been shown to be functional either through genomic integration or on replicating plasmids including chloramphenicol, hygromycin, kanamycin, tetracycline, and spectinomycin (145,146). A counterselectable marker, *sacB*, has been used successfully in this organism (147) and reporter genes such as GFP and *lacZ* have been used to visually screen *D. radiodurans* transformants as well (143,148).

Transformation to *D. radiodurans* was first demonstrated by Moseley and Setlow in 1968 (149). *D. radiodurans* has since been transformed with exogenous DNA using natural transformation (150), chemical transformation (151), and electroporation (152). It has been shown that the R-M systems of *D. radiodurans* affect the transformation efficiency of this species. Transformation of *D. radiodurans* with the same plasmid isolated from *E. coli* and from *D. radiodurans* resulted in a 20,000-fold decrease in transformation efficiency for the *E. coli* isolated plasmid (151). Similarly, Meima and Lidstrom transformed *D. radiodurans* with plasmids isolated from methylation-proficient and -deficient strains of *E. coli* and found that unmethylated plasmids resulted in 50-750-fold improvements in transformation efficiency (148). The authors therefore suggested that methylation-specific nucleases are likely restricting the transformed DNA, and ultimately affecting the transformation efficiency. They propose that the deletion of active R-M systems could lead to an improved *D. radiodurans* chassis for genetic engineering (148).

The multicopy genome of *D. radiodurans* can affect engineering efforts as genomic changes need to be made across all copies of the genome. A mutant library of *D. radiodurans* was built using Tn5 transposon-based random mutagenesis, providing a useful selection of strains for phenotypic or genetic screening (153). Due to *D. radiodurans* highly efficient recombination machinery, many of the successful targeted genome engineering efforts have used homologous recombination-based strategies. The first *D. radiodurans* genomic mutant was created by Smith in 1988 this way (154), followed by other examples through integration of linear DNA constructs or nonreplicative plasmids into the genome (143,155–157). Another targeted engineering method that has been developed for this organism is a Cre-*lox* recombinase system (158). CRISPR-based targeted engineering has not yet been demonstrated in *D. radiodurans*, though an experiment testing the toxicity of codon-optimized Cas9 and dCas9 on *D. radiodurans* cells showed high levels of toxicity (159). The reason this may not have been successfully demonstrated yet might be due to the limitations in engineering in polyploid organisms (160).

1.6.3 Metabolic properties and stress tolerance

There is an interest in microbial chassis that can utilize alternative feedstocks like lignocellulose from plant biomass as a carbon source since it is an abundant renewable resource (161). One reason that traditional chassis are not able to utilize these sources naturally is that they are not tolerant to lignocellulosic hydrolysate inhibitors that are released during pre-treatment or breakdown. Unlike traditional chassis, *D. radiodurans* is able to use this alternative feedstock as a source of energy, making it an attractive candidate chassis to enable sustainable bioproduction (162). In addition, some metabolomic and proteomic profiling has been performed in this species, usually in the context of the effect of harsh conditions (163,164); however, more intensive metabolic modelling is necessary as gaps in knowledge can make engineering this organism more difficult.

D. radiodurans is an extremophilic bacterium, meaning that it is able to survive in extreme conditions, and has been isolated from a range of natural environments. *D. radiodurans* has demonstrated resistance to many conditions including radiation (*e.g.*, ultraviolet, ionizing), oxidative stress, desiccation, high ethanol concentrations, and so on (165–167). The extreme resistance of *D. radiodurans* is multifactorial and is proposed to be due to mechanisms such as trehalose production (168) and the *irrE* gene, a transcriptional regulator of the pentose phosphate pathway (169). Cloning of this gene from *D. radiodurans* into traditional (*i.e.*, *E. coli, S. cerevisiae*) or non-traditional chassis improves their tolerance to stress conditions, namely to lignocellulosic hydrolysates, ethanol, butanol, acetate and acid (170–176). Other contributing factors may include the polyploid nature of the bacterium (177), the thick cell envelope (178), efficient DNA repair and recombination machinery (179), and manganese ion accumulation (180). The ability for *D. radiodurans* to withstand these harsh physiological conditions has permitted its survival in unique environments like the vacuum of space and has important implications for industrial processes.

1.6.4 Current and future applications

Overall, the high resistance of *D. radiodurans* to various stressors combined with its ability to utilize simple and sustainable carbon sources makes it an attractive chassis bacterium for many synthetic biology and industrial applications (56). Growth can be industrially scaled up using bioreactors, which has been previously demonstrated using a 10-L bioreactor for mixed nuclear waste remediation (181). In fact, *D. radiodurans* has shown a lot of promise for bioremediation of metals, organopollutants, toluene and mercury to name a few (155,157,182). It is also of interest as a chassis in the health industry for the development of human therapeutics in anti-aging and cancer research, as well as antioxidant biosynthesis (165,183–185). Additionally, a *D. radiodurans* strain has been created for the production of pinene, which is commonly used in the production of fragrances, pharmaceuticals, and biofuels (186).

D. radiodurans is also being investigated for use in the production of sustainable biomaterials such as concrete. Bacteria that are embedded in concrete, resulting in what is termed “living” or “self-healing” concrete, can repair cracks that form over time due to shrinkage through the precipitation of calcium carbonate crystals, which is the main component of limestone and cement (187,188). *D. radiodurans* was shown to be viable for up to 28 days in concrete, providing significant crack healing and increasing the compressive strength of the mortar by 42% at room temperature and 38% at near freezing temperatures (189). This is applicable to terrestrial construction, but also has implications for in-space manufacturing as well. Due to the ability to survive in space, this bacterium has made a name for itself in the aerospace industry and has even survived in exposed space conditions for up to 3 years outside the International Space Station (163,164,190,191). Finally, as a bacterial host for synthetic biology, researchers are interested in exploiting the molecular mechanisms of *D. radiodurans* such as efficient homologous recombination and DNA repair machinery with possible applications for DNA assembly.

1.7 Scope of the thesis

The cornerstone of this thesis is to further develop *S. meliloti* and *D. radiodurans* as bacterial chassis for synthetic biology through the expansion of their genetic toolkits. Given the prospective uses of these species for synthetic biology applications in agriculture, bioremediation, health and manufacturing, the generation of tools to allow further study and engineering of their genomes would be valuable. At the onset of my doctoral work, I sought to develop GC-rich chassis organisms with the intent on expanding the availability of microbial chassis beyond *E. coli* and *S. cerevisiae*.

I first set out to develop tools that would further the utility of *S. meliloti* as a bacterial chassis. In Chapter 2, I aimed to improve the transformation methods for introducing DNA into a reduced genome strain of *S. meliloti* using PEG-mediated transformation, electroporation and conjugation methods. I developed *S. meliloti* as a conjugative donor through the introduction of a conjugative helper plasmid and, using multi-host shuttle vectors, demonstrated for the first time the ability for *S. meliloti* to directly transfer DNA to *S. cerevisiae* and *P. tricornutum*. I characterized the conjugation frequency of vectors from *S. meliloti* to *E. coli*, *S. cerevisiae* and *P. tricornutum* and analyzed the occurrence of plasmid mutations and/or rearrangements when transferring to each recipient organism.

Then, I turned my efforts to furthering the development of *D. radiodurans* as a bacterial chassis. In Chapter 3, I adapted a multi-host shuttle vector from Chapter 2 with genetic parts for replication and selection in *D. radiodurans*. I showed the difficulty in transforming plasmid DNA isolated from *E. coli* into *D. radiodurans* and demonstrated conjugation from an *E. coli* donor as an efficient alternative. Taking advantage of *D. radiodurans*' natural propensity for homologous recombination, I developed a conjugation-based method for creating gene knockouts through conjugative delivery of non-replicating plasmids. Lastly, I cloned the large 78 kb MP1 megaplasmid from *D. radiodurans in vivo* using a non-replicative plasmid and subsequently cloned it into *E. coli*.

Finally in Chapter 4, I developed a method for generating seamless gene deletions in *D. radiodurans.* I demonstrated this method by sequentially targeting the known

restriction-modification systems of *D. radiodurans*, seamlessly deleting four out of six systems, and knocking out the fifth with a selective marker. The growth rate of the knockout strains was compared to the wild-type strain to determine if there were any negative effects on the fitness of *D. radiodurans* as a result of the gene deletions. These strains were then tested by chemical transformation of a small plasmid isolated from *E. coli* to determine if deletion of these restriction systems led to an increase in transformability. Ultimately, the generation of the genetic tools for *S. meliloti* and *D. radiodurans* should improve their utility as bacterial chassis for synthetic biology applications and foundational research.

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Chapter 2

2 Designer *Sinorhizobium meliloti* strains and multi-functional vectors enable direct inter-kingdom DNA transfer

The work presented in this chapter is reproduced (with permission – Appendix A) from:

Brumwell, SL, MacLeod, MR, Huang, T, Cochrane, RR, Meaney, RS, Zamani, M, ... & Karas, B. J. (2019). Designer *Sinorhizobium meliloti* strains and multi-functional vectors enable direct inter-kingdom DNA transfer. PloS one, 14(6), e0206781.

2.1 Introduction

The field of synthetic biology aims to utilize existing or novel biological parts and systems to create organisms that can help address global problems including increased demand for food, fuel, therapeutics, and high-value chemicals. However, one of the major obstacles in synthetic biology is that many organisms of interest lack genetic tools such as autonomously replicating plasmids, well-characterized promoters and terminators, selective markers, genome-editing tools, and protocols to uptake and install DNA (1,2). This problem can be addressed by cloning whole chromosomes or large DNA fragments from an organism of interest in a surrogate host, where genetic tools are in place and manipulations can be performed (3–5). Currently, the most common host for the capture and manipulation of DNA fragments is *Saccharomyces cerevisiae* (3,6–9). However, returning cloned or engineered fragments to destination cells is still challenging due to the lack of direct transfer methods from *S. cerevisiae*, such as bacterial conjugation. In addition, *S. cerevisiae* cannot maintain large DNA fragments with G+C content >40% without additional engineering (10–12), and many bacterial strains of industrial interest have a G+C content above this range. For example, *Streptomyces* species have a G+C content >65% and are important for the production of antibiotics (gentamicin, kanamycin, tetracycline, etc.) (13). Therefore, it is desirable to develop a

host to clone, maintain, manipulate, and transfer DNA fragments, including those with high G+C content, to bacterial and eukaryotic destination cells.

Sinorhizobium meliloti is an attractive host candidate for this application. *S. meliloti* is a Gram-negative α-Proteobacterium that forms a symbiotic relationship with legume plants where it fixes nitrogen in root nodules, and therefore is highly important in agriculture. *S. meliloti* model strain Rm1021 has a multipartite genome including a chromosome (3.65 Mb), pSymA megaplasmid (1.35 Mb), and pSymB chromid (1.68 Mb) (14). Multiple derivatives of *S. meliloti* now exist that vary in genome size, nutrient requirements and generation time (15). Recently, a derivative of *S. meliloti* with a minimal genome lacking the pSymA and pSymB replicons was developed, resulting in a 45% reduction of the genome (15). Two essential genes were identified in the pSymB chromid, *engA* and $tRNA^{arg}$, and these genes were transferred to the main chromosome (16). The replication origins of pSymA and pSymB were identified and characterized (17), and could be used to generate designer replicating plasmids. Additionally, several genetic tools have been developed for this species including vectors based on *repABC* origins taken from various α-Proteobacteria that can be propagated in *S. meliloti* (18). Three of these vectors can be maintained within wild-type *S. meliloti* at one time, along with the endogenous pSymA and pSymB replicons. Utilizing these vectors, an *in vivo* cloning method with Cre/lox-mediated translocation of large DNA fragments to the *repABC*-based vector was established (18). Therefore, *S. meliloti* is an attractive host organism due to its high G+C content genome (62%) (19), available origins of replication that could be used to maintain large DNA fragments, expanding genetic toolbox, and applications in agriculture.

S. meliloti is also an attractive host bacterium because it is able to move DNA to other organisms using several methods. Previous studies have demonstrated DNA transfer methods from *S. meliloti* through introduction of a Ti plasmid to plant species through mobilization of TDNA (20). In addition, *S. meliloti* is able to move DNA to bacteria via conjugation in tri-parental mating experiments (18). Installing a conjugative plasmid into *S. meliloti*, such as pTA-Mob (21), will allow for faster and more efficient transfer of DNA to a recipient organism. The conjugative pTA-Mob plasmid, which

contains all of the genes required for direct DNA transfer via conjugation, can mobilize any cargo plasmid containing an origin of transfer (*oriT*). Conjugation is a particularly attractive method of DNA transfer to streamline the workflow for genetic manipulation of organisms. While transfer of DNA from *S. meliloti* to bacterial cells has been demonstrated, DNA transfer to eukaryotic cells, such as yeast and microalgae, has not been previously described.

Here, we utilized the reduced-genome strains of *S. meliloti* (15) to create designer strains with the restriction-system removed and the conjugative pTA-Mob plasmid installed. In addition, we used identified origins from *S. meliloti* pSymA and pSymB replicons to create multi-host shuttle (MHS) vectors that replicate in *S. meliloti, Escherichia coli, S. cerevisiae,* and *Phaeodactylum tricornutum.* The MHS vectors were tested for their ability to provide antibiotic resistance and propagate in *S. meliloti.* These vectors were moved into *S. meliloti* strains via i) an optimized electroporation protocol, ii) a newly developed polyethylene glycol (PEG) method, or iii) conjugation from *E. coli*. Most notably, we have developed protocols to directly transfer the MHS vectors via conjugation from *S. meliloti* to *E. coli, S. cerevisiae*, and *P. tricornutum*.

2.2 Results and Discussion

2.2.1 Development of designer bacterial strains

Initially, we engineered *S. meliloti* strains to remove the restriction-system (Δ*hsdR*) to allow for development of more efficient transformation methods. Disruption or deletion of the *hsdR* gene has been previously reported in the Rm1021 strain of *S. meliloti,* and deletion mutants were shown to have enhanced transformation efficiencies (22,18). In our reduced-genome *S. meliloti* strains, either lacking one or both of the pSymA or pSymB replicons, the *hsdR* gene was replaced by an FRT-Km/Nm-FRT cassette and the resulting Δ*hsdR*::Nm mutant allele was transferred to various background strains via transduction of Nm^R. The FRT-Km/Nm-FRT cassette was then removed by introducing an unstable plasmid (pTH2505) carrying the Flp recombinase, followed by curing of this plasmid. The *hsdR* deletion strains were verified to have the *hsdR* gene successfully deleted using

diagnostic PCR with primers located upstream and downstream of the *hsdR* gene locus (Supplemental Figure B-1). *S. meliloti* RmP4098 ΔpSymA Δ*hsdR*, retaining the Km/Nm cassette and the pSymB chromid, was chosen as our host strain as it had the fastest doubling time when compared to the other reduced-genome strains (15). *S. meliloti* ΔpSymA Δ*hsdR* was transformed with the pTA-Mob conjugative helper plasmid (21) and used as the conjugative donor strain for all subsequent experiments (Figure 2-1a). In addition, reduced-genome *S. meliloti* strains RmP3952 ΔpSymB Δ*hsdR* and RmP3909 ΔpSymAB Δ*hsdR* were developed using the same method described above.

A designer *E. coli* strain was developed to simplify the current method of conjugation from *E. coli* to *S. meliloti* (Figure 2-1b). We used lambda red recombination to create an auxotrophic strain of *E. coli* Epi300, named ECGE101, by deleting the *dapA* gene. This gene is required for synthesis of diaminopimelic acid (DAP) (23), which is necessary for production of the bacterial cell wall. Therefore, ECGE101 requires DAP supplementation in the growth media and provides a useful, antibiotic-free method for counter-selecting *E. coli* after conjugation to *S. meliloti* or any other organism.

2.2.2 Design, assembly and characterization of multi-host shuttle vectors

With the reduced, restriction-system minus strains of *S. meliloti* created, and the origins of replications of the large megaplasmid and chromid (*repA1B1C1* and *repA2B2C2*) identified, MHS vectors were developed as cargo plasmids for conjugation from *S. meliloti* to various recipients. Six MHS vectors were designed and constructed to allow for stable replication and selection in our *S. meliloti* conjugative donor, as well as several conjugative recipient organisms including *E. coli*, *S. cerevisiae*, and *P. tricornutum*. These organisms were chosen as conjugative recipients as they are well-characterized model strains for bacterial, yeast and algal systems.

These vectors were constructed with a bacterial artificial chromosome (BAC) and yeast artificial chromosome (YAC) backbone allowing for replication in *E. coli* and *S.*

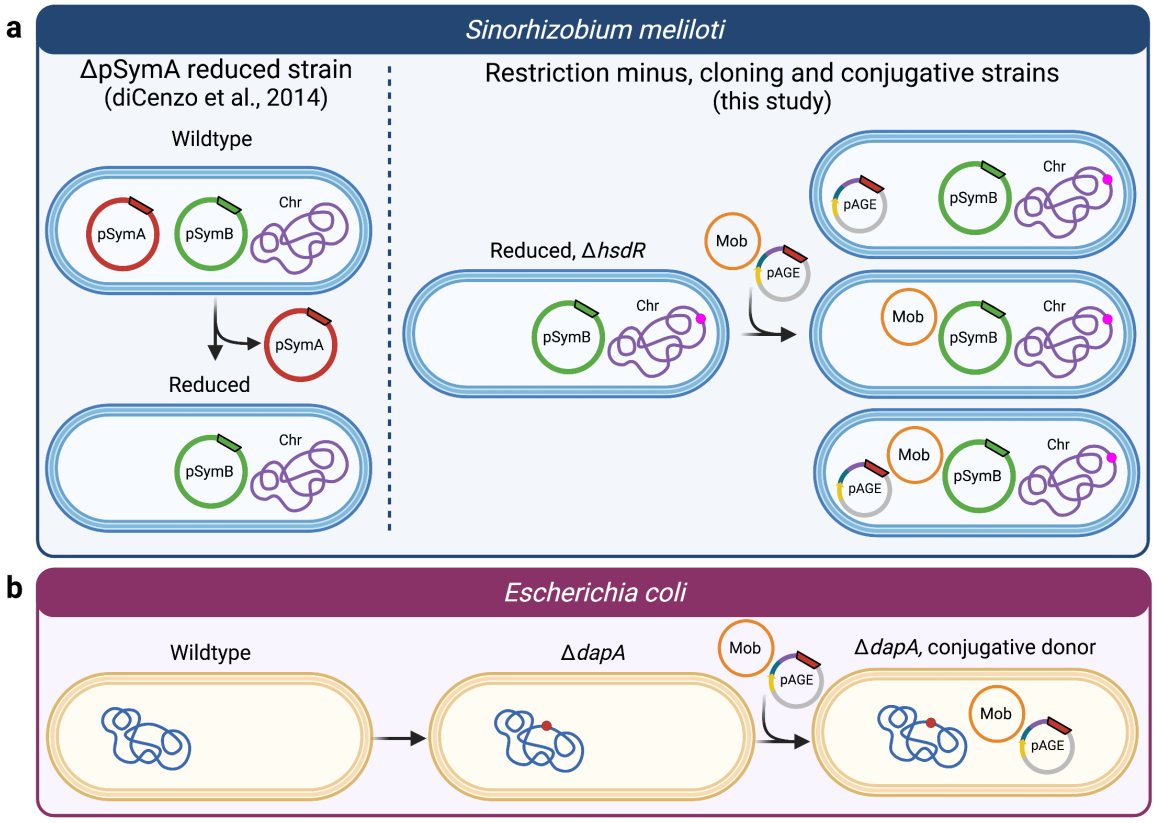

Figure 2-1. Development of designer *S. meliloti* ΔpSymA Δ*hsdR* and *E. coli* Δ*dapA* strains. (a) Simplified schematic for the creation of (left) the genome reduced *S. meliloti* strain lacking the pSymA megaplasmid (15) and (right) *S. meliloti* ΔpSymA strains following deletion of the *hsdR* restriction-system, and harbouring either a pAGE plasmid, pTA-Mob conjugative plasmid or both. (b) Simplified schematic of *E. coli* engineering to create a Δ*dapA* strain and conjugative donor strain harbouring pTA-Mob and a pAGE plasmid. Created with BioRender.com.

cerevisiae (24). The YAC also allows replication in *P. tricornutum* (24,25). The MHS vectors contain an origin of replication captured from the native megaplasmid of *S. meliloti*, pSymA (*repA2B2C2*, denoted pAGE vectors), or the pSymB chromid (*repA1B1C1*, denoted pBGE vectors). Selectable markers include spectinomycin (Sp), tetracycline (Tc), or kanamycin/neomycin (Km/Nm) resistance for *S. meliloti* (but also conferring some resistance in *E. coli*), nourseothricin N-acetyl transferase (Ntc) for *P. tricornutum* (26), HIS3 for *S. cerevisiae*, and chloramphenicol (Cm) resistance for *E. coli*. Finally, all MHS vectors contain an origin of transfer (*oriT*) that is acted on by proteins encoded on the conjugative plasmid pTA-Mob, and is necessary for mobilization of the MHS vectors from *S. meliloti* to recipient organisms (Figure 2-2a). Three versions of the pAGE vector differing only in the *S. meliloti* selective marker, pAGE1.0 (Sp), pAGE2.0 (Tc), and pAGE3.0 (Km/Nm), were constructed with the pSymA origin (*repA2B2C2*). An additional three pBGE vectors differing only in the *S. meliloti* selective marker, pBGE1.0 (Sp), pBGE2.0 (Tc), and pBGE3.0 (Km/Nm), were constructed with the pSymB origin (*repA1B1C1*). Once assembled (27), the vectors were transformed into *E. coli* Epi300 cells, isolated and confirmed to be correctly assembled by a diagnostic restriction digest (Figure 2-2b).

2.2.3 Optimization of DNA transfer to *S. meliloti* via electroporation, a new polyethylene glycol transformation method and conjugation

In order to develop *S. meliloti* as a host, a highly efficient transformation method is required for the uptake of DNA. As such, we sought to introduce the pAGE vectors into *S. meliloti* ΔpSymA Δ*hsdR* Nm^s using conjugation, electroporation, and PEG-mediated transformation (Figure 2-3a). Conjugation frequency and transformation efficiency (for electroporation and PEG-mediated transformation) was determined using one or more of the pAGE vectors (Figure 2-3b). Currently, the most common transformation method used in *S. meliloti* is electroporation. Optimization of this method through transformation of *S. meliloti* with three pAGE vectors (~18 kb) produced efficiencies averaging 1.4 x 10^5 CFU μg of DNA^{-1} (Figure 2-3b, Supplemental Figure B-2).

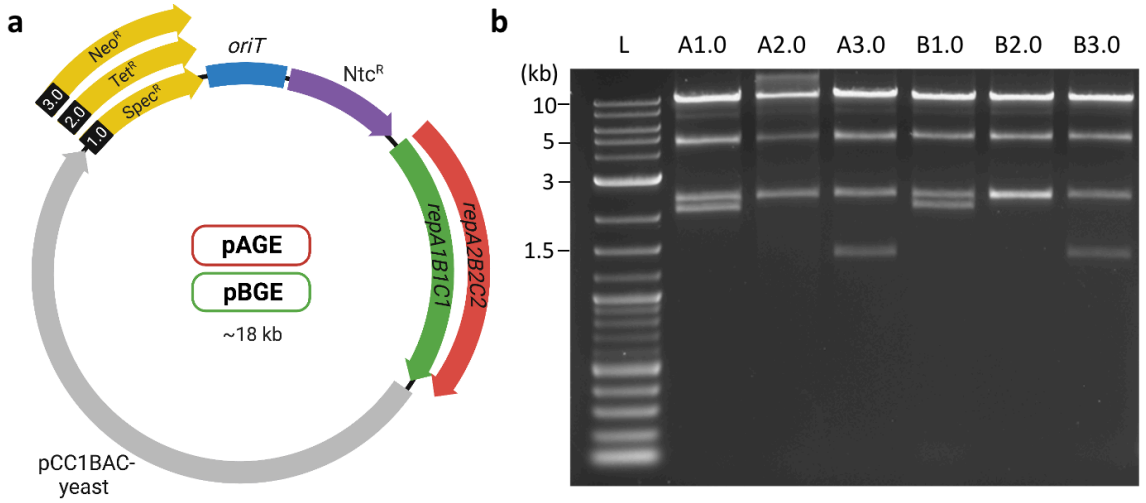

Figure 2-2. Creation and analysis of multi-host shuttle vectors containing pSymA and pSymB origins of replication. (a) Map of pAGE/pBGE multi-host shuttle vector design with both standard and interchangeable components. Standard components include the BAC and YAC backbone (pCC1BAC-yeast), an origin of transfer, and the *P. tricornutum* selectable marker nourseothricin N-acetyl transferase (*nat*). Interchangeable components include selectable markers for *S. meliloti*: spectinomycin (1.0 - Spec), tetracycline (2.0 - Tet), or neomycin (3.0 - Neo); and *S. meliloti* origin of replication: the pSymA origin (*repA2B2C2*) or pSymB origin (*repA1B1C1*). Created with BioRender.com. (b) Diagnostic restriction digest of pAGE (A1.0-3.0) and pBGE (B1.0-3.0) vectors following assembly in yeast and induction to high copy number in *E. coli*, with I-CeuI, I-SceI, PacI, and PmeI. Expected band sizes (in bp) for pAGE1.0 and pBGE1.0 are 9395, 4361 (A only), 4523 (B only), 2298, 2075; for pAGE2.0 and pBGE2.0 are 9524, 4361 (A only), 4523 (B only), 2298, 2294; for pAGE3.0 and pBGE3.0 are 9524, 4361 (A only), 4523 (B only). L, 2-log ladder.

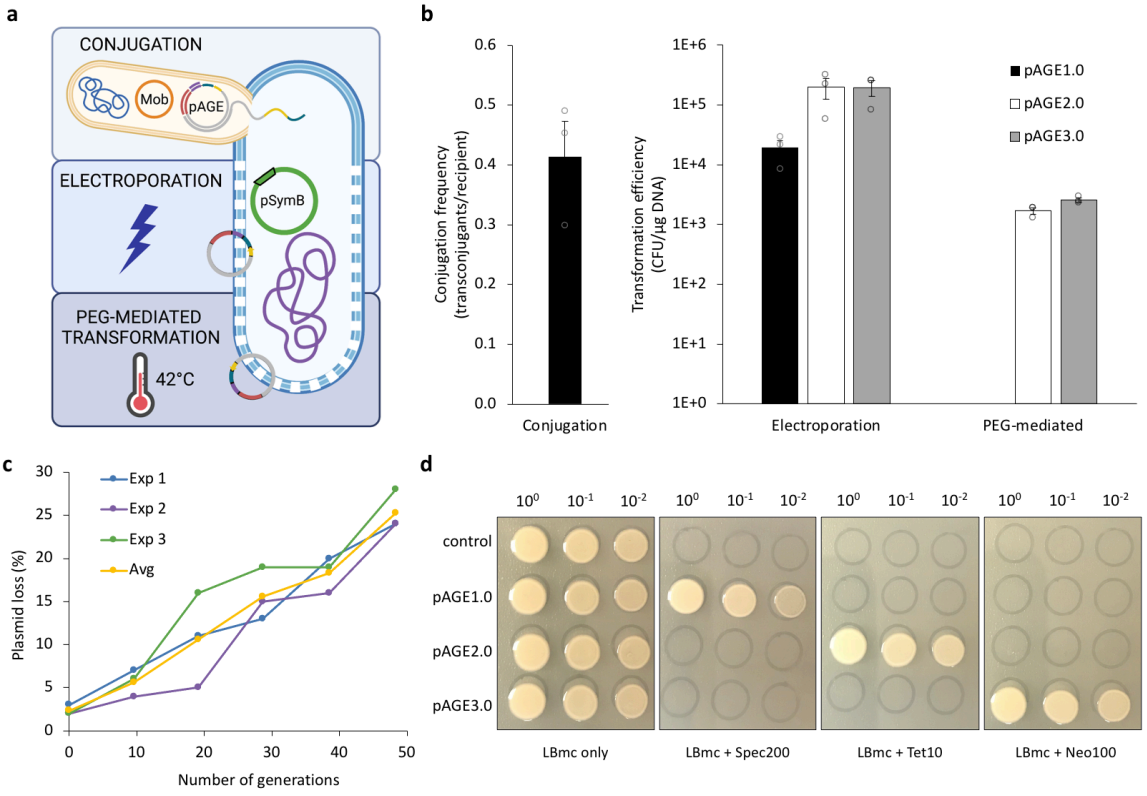

Figure 2-3. DNA transfer of pAGE vectors to *S. meliloti* RmP4122 ΔpSymA Δ*hsdR*. (a) Depiction of pAGE plasmid transfer to *S. meliloti* using three methods: conjugation from an *E. coli* ECGE101 pTA-Mob (Mob) donor, electroporation and PEG-mediated transformation. Created with BioRender.com. (b) Conjugation frequency (reported as transconjugants per recipient) or transformation efficiency (reported as CFU μg^{-1} of DNA on a log10 base scale) of pAGE1.0, pAGE2.0 or pAGE3.0 plasmids to *S. meliloti*. Three biological replicates were performed for each condition and each point represents the average of three technical replicates. Error bars represent standard error of the mean. Note: conjugation was only tested with pAGE1.0 and PEG-mediated transformation of pAGE1.0 consistently resulted in a similar number of colonies on experimental and control plates, therefore this was not included (refer to Supplemental Figure B-4). (c) Vector stability assay of pAGE2.0 in *S. meliloti* grown in nonselective media (LBmc 38 µM $FeCl_3$) over 50 generations. Every 10 generations, the cultures were plated on nonselective media and selective media supplemented with tetracycline 5 µg mL^{-1}. The percentage of *S. meliloti* colonies on nonselective media that were unable to grow on selective media was used as an indication of vector stability. Three biological replicates were performed and the average of these replicates is plotted. (d) Following conjugation of pAGE1.0, pAGE2.0 and pAGE3.0 from *E. coli* to *S. meliloti*, one *S. meliloti* transconjugant harbouring each plasmid was plated on nonselective media and media supplemented with either spectinomycin 200 µg mL^{-1}, tetracycline 10 µg mL^{-1} or neomycin 100 µg mL^{-1} along with *S. meliloti* without a plasmid (control).

Following transformation of pAGE2.0 into *S. meliloti* by electroporation, vector stability was tested by iterative subculturing every 10 generations to a total of about 50 generations. We observed, on average, that approximately 25% of vectors were lost after 50 generations, as determined by the number of colonies unable to grow on selective media (LBmc 38 μM $FeCl_3$ 2 μM $CoCl_2$ Tc 5 μg mL^{-1}) after restreaking from non-selective media (LBmc 38 μM $FeCl_3$ 2 μM $CoCl_2$) (Figure 2-3c). Additionally, since a PEG-mediated transformation method was successfully applied to move large DNA fragments (>1 Mb) in the transplantation protocol used to create the first synthetic cell (4), we developed a PEG-mediated transformation method in *S. meliloti* and were able to obtain efficiencies on average of 2.1 x 10^3 CFU μg^{-1} of DNA (Figure 2-3b, Supplemental Figure B-3 and B-4). In addition, conjugation has been previously established as a method of DNA transfer to *S. meliloti* (18,28); therefore, we have developed an improved conjugation protocol from our conjugative *E. coli* ECGE101 strain carrying the pTA-Mob and pAGE1.0 vectors. Using this method, we obtained a conjugation efficiency averaging 4 transconjugants per every 10 recipient cells (*i.e.,* 0.4 transconjugants/recipient) (Figure 2-3b). The three pAGE vectors were then conjugated to *S. meliloti* by *E. coli* ECGE101 conjugative strains, and *S. meliloti* transconjugants were plated on media supplemented with their respective antibiotic selections. We demonstrated that pAGE1.0, pAGE2.0, and pAGE3.0 in *S. meliloti* provide resistance to Sp, Tc, and Nm, respectively (Figure 2-3d).

2.2.4 Direct DNA transfer via conjugation from *S. meliloti* to *E. coli*, *S. cerevisiae* and *P. tricornutum*

We utilized the designer *S. meliloti* RmP4098 ΔpSymA Δ*hsdR* host strain carrying the pTA-Mob conjugative plasmid and pAGE1.0 compatible genome engineering vector to develop direct DNA transfer of pAGE1.0 via conjugation from *S. meliloti* to *E. coli, S. cerevisiae,* and *P. tricornutum* (Figure 2-4a-c,e). The ~18 kb pAGE1.0 vector contains the pSymA origin (*repA2B2C2*) and the spectinomycin selectable marker for *S. meliloti*. The number of *S. meliloti* donor cells to each recipient cell type was ~2:1 for *E. coli*, 26:1 for *S. cerevisiae* and 74:1 for *P. tricornutum* (Figure 2-5a). Conjugation efficiencies were

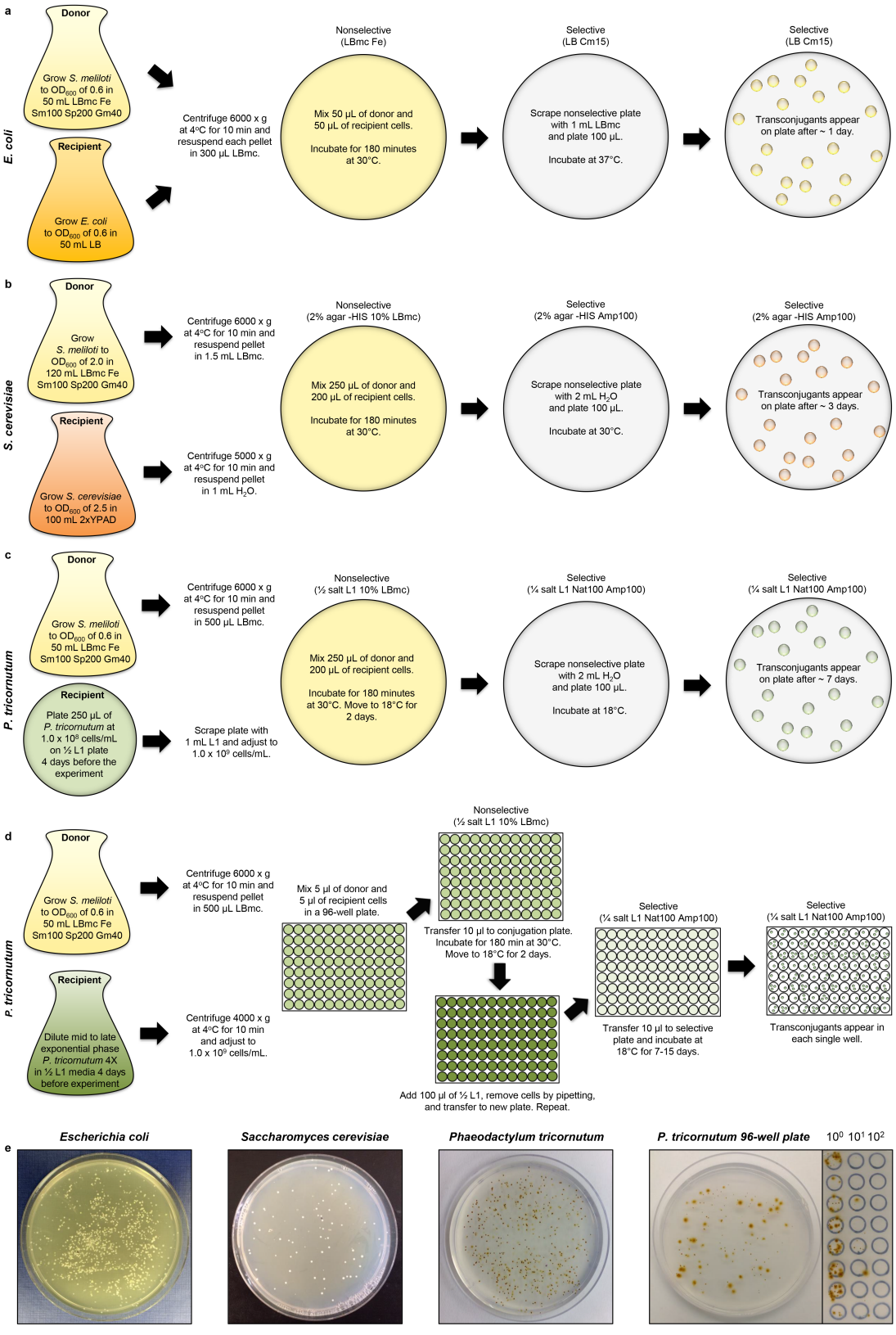
a
E. coli
Donor
Grow S. meliloti to OD600 of 0.6 in 50 mL LBmc Fe Sm100 Sp200 Gm40
Recipient
Grow E. coli to OD600 of 0.6 in 50 mL LB
Centrifuge 6000 x g at 4°C for 10 min and resuspend each pellet in 300 µL LBmc.
Nonselective (LBmc Fe)
Mix 50 µL of donor and 50 µL of recipient cells.
Incubate for 180 minutes at 30°C.
Selective (LB Cm15)
Scrape nonselective plate with 1 mL LBmc and plate 100 µL.
Incubate at 37°C.
Selective (LB Cm15)
Transconjugants appear on plate after ~ 1 day.
b
S. cerevisiae
Donor
Grow S. meliloti to OD600 of 2.0 in 120 mL LBmc Fe Sm100 Sp200 Gm40
Recipient
Grow S. cerevisiae to OD600 of 2.5 in 100 mL 2xYPAD
Centrifuge 6000 x g at 4°C for 10 min and resuspend pellet in 1.5 mL LBmc.
Centrifuge 5000 x g at 4°C for 10 min and resuspend pellet in 1 mL H2O.
Nonselective (2% agar -HIS 10% LBmc)
Mix 250 µL of donor and 200 µL of recipient cells.
Incubate for 180 minutes at 30°C.
Selective (2% agar -HIS Amp100)
Scrape nonselective plate with 2 mL H2O and plate 100 µL.
Incubate at 30°C.
Selective (2% agar -HIS Amp100)
Transconjugants appear on plate after ~ 3 days.
c
P. tricornutum
Donor
Grow S. meliloti to OD600 of 0.6 in 50 mL LBmc Fe Sm100 Sp200 Gm40
Recipient
Plate 250 µL of P. tricornutum at 1.0 x 10^8 cells/mL on ½ L1 plate 4 days before the experiment
Centrifuge 6000 x g at 4°C for 10 min and resuspend pellet in 500 µL LBmc.
Scrape plate with 1 mL L1 and adjust to 1.0 x 10^9 cells/mL.
Nonselective (½ salt L1 10% LBmc)
Mix 250 µL of donor and 200 µL of recipient cells.
Incubate for 180 minutes at 30°C. Move to 18°C for 2 days.
Selective (¼ salt L1 Nat100 Amp100)
Scrape nonselective plate with 2 mL H2O and plate 100 µL.
Incubate at 18°C.
Selective (¼ salt L1 Nat100 Amp100)
Transconjugants appear on plate after ~ 7 days.
d
P. tricornutum
Donor
Grow S. meliloti to OD600 of 0.6 in 50 mL LBmc Fe Sm100 Sp200 Gm40
Recipient
Dilute mid to late exponential phase P. tricornutum 4X in ½ L1 media 4 days before experiment
Centrifuge 6000 x g at 4°C for 10 min and resuspend pellet in 500 µL LBmc.
Centrifuge 4000 x g at 4°C for 10 min and adjust to 1.0 x 10^9 cells/mL.
Mix 5 µl of donor and 5 µl of recipient cells in a 96-well plate.
Nonselective (½ salt L1 10% LBmc)
Transfer 10 µl to conjugation plate. Incubate for 180 min at 30°C. Move to 18°C for 2 days.
Add 100 µl of ½ L1, remove cells by pipetting, and transfer to new plate. Repeat.
Selective (¼ salt L1 Nat100 Amp100)
Transfer 10 µl to selective plate and incubate at 18°C for 7-15 days.
Selective (¼ salt L1 Nat100 Amp100)
Transconjugants appear in each single well.
e
Escherichia coli
Saccharomyces cerevisiae
Phaeodactylum tricornutum
P. tricornutum 96-well plate
10^0 10^1 10^2

Figure 2-4. Optimized conjugation protocols from *S. meliloti* to *E. coli*, yeast and microalgae. Schematic of protocol for conjugation of pAGE1.0 from *S. meliloti* to (a) *E. coli*, (b) *S. cerevisiae,* (c) *P. tricornutum* – standard protocol, and (d) *P. tricornutum* – 96-well plate protocol. Note: ¼ salt L1 plates were made with ¼ aquil salts concentration but all other components are the same as would be used for ½ L1 plates (e) Examples of plates containing *E. coli*, *S. cerevisiae*, and *P. tricornutum* (standard and in a 96-well plate) transconjugants. *P. tricornutum* transconjugants from the 96-well plate protocol are shown as 100 μL plated on a full plate, and 10 μL serially diluted on spot plates. Notes: Antibiotic concentrations are given in μg mL^{-1}. The *S. meliloti* RmP4098 ΔpSymA Δ*hsdR* strain used is resistant to streptomycin (100 μg mL^{-1}) and requires Fe supplementation (38 μM $FeCl_3$) for growth. The pAGE1.0 plasmid confers resistance to spectinomycin (200 μg mL^{-1}). Media supplementation may vary with chosen *S. meliloti* host and MHS vector.

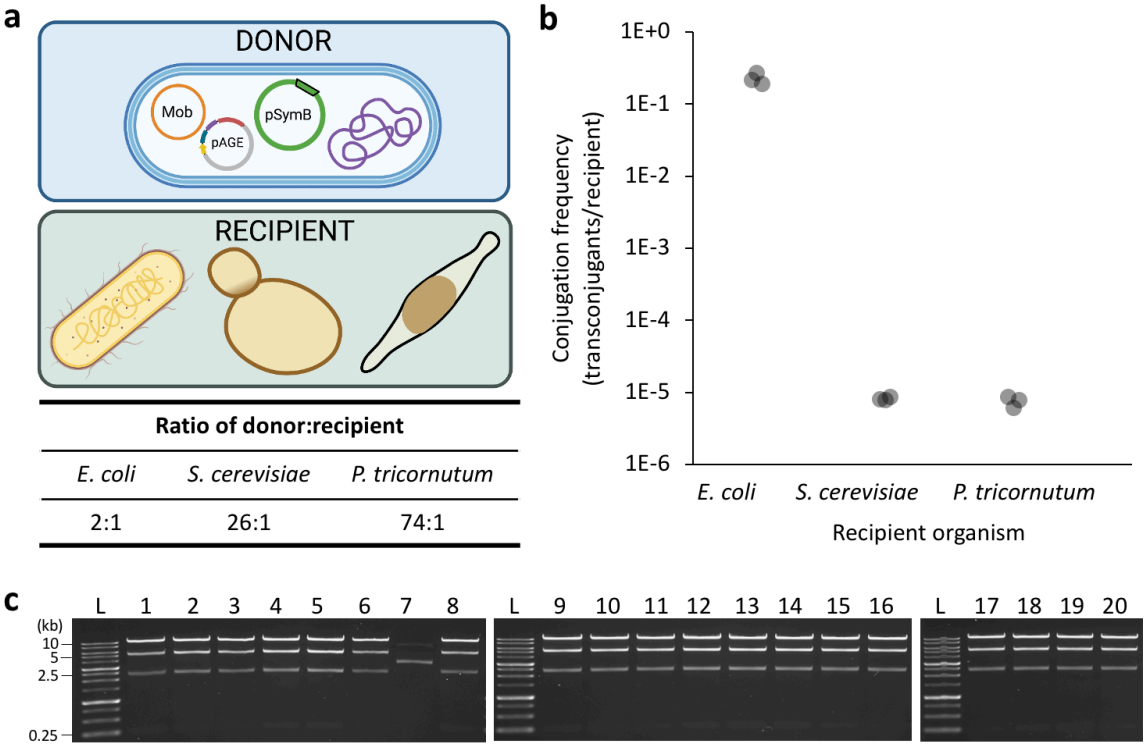

Figure 2-5. Conjugation of pAGE1.0 from *S. meliloti* donor to *E. coli*, yeast and microalgae recipients. (a) Diagram of the *S. meliloti* pTA-Mob (Mob) pAGE1.0 donor strain and *E. coli*, *S. cerevisiae* and *P. tricornutum* recipients. Created with BioRender.com. The ratio of donor to recipients used in each pairing is reported and was determined by pre-conjugation plating of donor and recipients. (b) Conjugation frequency (transconjugants/recipient) for each donor-recipient pair as determined by plating on selective plates and non-selective plates. Three biological replicates were performed and each point represents the average of three technical replicates. (c) Representative set of EcoRV-HF diagnostic restriction digests performed on pAGE1.0 plasmids isolated from 20 *S. cerevisiae* transconjugant colonies following conjugation from *S. meliloti*. Prior to digestion, plasmids were transformed into *E. coli*, induced to high copy number and reisolated. Expected band sizes (in bp) are 10,288, 5235, 2377, and 229 following gel electrophoresis on a 1% agarose gel. L, 1 kb ladder. 1-20, 20 individual pAGE1.0 vectors. Note: the 229 bp band is very faint but visible with increased exposure.

calculated as the number of transconjugants per recipient cell from the *S. meliloti* conjugative donor to *E. coli, S. cerevisiae*, and *P. tricornutum* as 2.22 x 10^{-1}, 7.99 x 10^{-6} and 7.42 x 10^{-6}, respectively (Figure 2-5b). Additionally, we developed a high throughput conjugation protocol in a 96-well plate from *S. meliloti* to *P. tricornutum* that could be used for large-scale experiments and in an automated facility (Figure 2-4d,e). Development of these direct DNA transfer protocols is a critical step in the development of *S. meliloti* as a robust host for genome engineering and will facilitate its use in synthetic biology applications.

Next, we evaluated the conjugated pAGE1.0 vectors for DNA rearrangements and potential mutations that could have been introduced during conjugation from *S. meliloti* to *E. coli*, *S. cerevisiae* and *P. tricornutum*. In the first case, 20 *E. coli* transconjugant vectors were induced with 0.1% arabinose to obtain high copy number and were directly isolated from *E. coli*. The pAGE1.0 vector replicates as a low-copy plasmid in *S. cerevisiae* and *P. tricornutum* and it cannot be induced with arabinose to obtain high copy number within these species. Therefore, 60 vectors from *S. cerevisiae* and *P. tricornutum* transconjugants were isolated and transformed into *E. coli* to obtain high quality DNA. DNA isolated from 59 out of 60 *S. cerevisiae* transconjugants and 58 out of 60 *P. tricornutum* transconjugants resulted in *E. coli* colonies following transformation (Table 2-1). Then, 20 *E. coli* transformants (each transformed with DNA from independent *S. cerevisiae* colonies) and 30 *E. coli* transformants (each transformed with DNA from independent *P. tricornutum* colonies) were selected, induced with 0.1% arabinose, and the vector DNA was isolated. Diagnostic restriction digests were performed on these vectors using EcoRV-HF, and the number of vectors with the expected banding pattern was 18/20 originating from *E. coli*, 19/20 originating from *S. cerevisiae* and 16/30 originating from *P. tricornutum* (Figure 2-5c, Supplemental Figure B-5 and B-6). Therefore, we observed that correct pAGE1.0 vectors can be rescued when conjugated from *S. meliloti* 90% of time in *E. coli*, 93% of the time in *S. cerevisiae*, and 52% of the time in *P. tricornutum* (Table 2-1).

Finally, whole-plasmid sequencing was performed on pAGE1.0 plasmids recovered from two *E. coli*, *S. cerevisiae* and *P. tricornutum* transconjugants that had a

Table 2-1. Analysis of pAGE1.0 vectors recovered from *E. coli, S. cerevisiae,* and *P. tricornutum* transconjugants. Following conjugation, 60 pAGE1.0 vectors from *S. cerevisiae* and *P. tricornutum* transconjugants were isolated and transformed into *E. coli.* The number of transformations resulting in *E. coli* colonies, out of 60, and the average number of *E. coli* colonies was determined. Then, plasmids from 20 *E. coli* colonies, including the *E. coli* transconjugants, were induced with arabinose and once again isolated to obtain high quality DNA. Diagnostic restriction digests of the pAGE1.0 vectors were performed with EcoRV-HF and the number that were correct out of 20 were reported. The likelihood of obtaining correct vectors based on the number of plasmids able to be rescued in *E. coli* and the subsequent number correct by digest is reported as a percentage. Note: some transformations of pAGE1.0 plasmids from *P. tricornutum* transconjugants to *E. coli* resulted in a very small number of colonies (~5), therefore these were grouped as transformations resulting in <25 and >25 colonies. Consequently, a diagnostic restriction digest was performed on 30 plasmids from *P. tricornutum* transconjugants.

Recipient	Number of vectors rescued in *E. coli*	Mean number of *E. coli* colonies	Number of vectors correct by digest	Likelihood of obtaining correct vectors
E. coli	n/a	n/a	18/20	90%
P. tricornutum	16/60 (<25 colonies)	5	0/10	56%
	42/60 (>25 colonies)	316	16/20	
S. cerevisiae	59/60	15	19/20	93%

correct banding pattern following digestion. Identified mutations summarized in Table 2-2 revealed that no mutations were introduced during conjugation to *E. coli* or *S. cerevisiae*. Two insertion mutations were introduced into each pAGE1.0 plasmid isolated from *P. tricornutum* transconjugants; these mutations were identified in non-coding regions including the plasmid backbone and the FcpD promoter. These results are consistent with previous studies that observed mutations in plasmids following conjugation from *E. coli* to *P. tricornutum* (24,26). In addition, 27% of *P. tricornutum* isolated plasmids transformed to *E. coli* produced less than 25 transformants, all of which contained plasmids with gross rearrangements observed by restriction digestion (Table 2-1). Combined, these results could be indicative of plasmid shearing during extraction from *P. tricornutum* transconjugants or a less stable connection during conjugation between bacteria and microalgae compared to the other recipient organisms.

In summary, pAGE and pBGE MHS vectors were developed and shown to be stably propagated in *S. meliloti*, *E. coli*, *S. cerevisiae*, and *P. tricornutum*. These multi-host and multi-functional vectors have promising applications for the cloning of large, high G+C content DNA fragments and their direct transfer to prokaryotic and eukaryotic target organisms. Conjugation of these vectors from *S. meliloti* to *E. coli*, *S. cerevisiae*, and *P. tricornutum*, as well as showing *S. meliloti* as a conjugative recipient, illustrates that *S. meliloti* can be a suitable host organism for inter-kingdom transfer of DNA. Due to the range of recipient organisms demonstrated, conjugation could be expanded to other target organisms in the future. These hosts will be invaluable for the cloning and installation of genes, synthetic chromosomes, or large biosynthetic pathways, the study of organisms lacking genetic tools, and specifically for applications in industrially and agriculturally important strains such as plants, marine organisms, and soil microbiomes.

2.3 Materials and Methods

2.3.1 Microbial strains and growth conditions

Sinorhizobium meliloti strains were grown at 30°C in LBmc medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, 0.301 g $MgSO_4$, and 0.277 g anhydrous $CaCl_2$ were

Table 2-2. Summary of mutations identified in plasmids isolated from *S. meliloti*, *E. coli*, *S. cerevisiae*, and *P. tricornutum* transconjugants. Two pAGE1.0 plasmids isolated from each transconjugant recipient were sequenced by next-generation whole plasmid sequencing at CCIB DNA Core (Massachusetts General Hospital, Boston, MA, USA). The consensus sequences were aligned to the original pAGE1.0 sequenced prior to conjugation using Benchling [Biology Software, 2019, https://benchling.com] and the number and location of any mutations are reported. Position 1 of the plasmid was reindexed to the start of the ClaI site directly upstream of the FcpD promoter.

Donor	Recipient	Plasmid Number	Number of Mutations	Mutation	Position
E. coli	*S. meliloti*	1	0	n/a	n/a
		2	0	n/a	n/a
S. meliloti	*E. coli*	1	0	n/a	n/a
		2	0	n/a	n/a
S. meliloti	*S. cerevisiae*	1	0	n/a	n/a
		2	0	n/a	n/a
S. meliloti	*P. tricornutum*	1	2	Insertion - A	11668 (pCC1BAC-yeast)
				Insertion - C	14826 (pCC1BAC-yeast)
		2	2	Insertion - A	553 (FcpD promoter)
				Insertion - C	13244 (pCC1BAC-yeast)

dissolved in sddH_2O to a final volume of 1 L, and then autoclaved. Solid plates contained 1.5% agar.) supplemented with appropriate antibiotics (streptomycin (100 μg mL^{-1}), spectinomycin (200 μg mL^{-1}), gentamicin (40 μg mL^{-1}), tetracycline (5 μg mL^{-1}), neomycin (100 μg mL^{-1})), and 38 μM $FeCl_3$ and/or 2 μM $CoCl_2$, when appropriate. *Saccharomyces cerevisiae* VL6−48 (ATCC MYA-3666: MATα *his*3-Δ200 *trp*1-Δ1 *ura*3−52 *lys*2 *ade*2−1 *met*14 cir^0) was grown at 30°C in rich medium (2X YPD) supplemented with 80 mg L^{-1} adenine hemisulfate, or yeast synthetic complete medium lacking histidine supplemented with 60 mg L^{-1} adenine sulfate (Teknova, Inc.) (8). *Escherichia coli* (Epi300, Epicenter) was grown at 37°C in Luria Broth (LB) supplemented with appropriate antibiotics (chloramphenicol (15 μg mL^{-1})). *Escherichia coli* (ECGE101) was grown at 37°C in Luria Broth (LB) supplemented with appropriate antibiotics (chloramphenicol (15 μg mL^{-1}), gentamicin (40 μg mL^{-1})), and DAP (60 μg mL^{-1}). *Phaeodactylum tricornutum* (Culture Collection of Algae and Protozoa CCAP 1055/1) was grown in L1 medium 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 (26). L1 media was prepared as previously described (26). The list of strains used and created in this study can be found in Supplemental Table B-1.

2.3.2 Development of Δ*hsdR S. meliloti* strains

Designer *S. meliloti* strains used in this study were created by taking the strain RmP3500, which lacks pSymA and pSymB where the *engA-tRNA^{Arg}-rmlC* genomic region has been introduced into the chromosome (29) and reintroducing either pSymA or pSymB or both. Specifically, RmP4098, was made by reintroducing pSymB from RmP3491 into the strain RmP3910, which is derived from RmP3500, to create the strain RmP3950. From there, a Nm resistance cassette from strain RmP3975 was transduced into strain RmP3950. Double homologous recombination of the Nm cassette from RmP3975 into the genome of RmP3950 was selected on Nm Sm plates. The resulting strain, RmP4098, is ΔpSymA pSymB+ *hsdR*::Nm, Nm^R Sm^R.

RmP3975 is a strain where the *hsdR* restriction gene has been replaced by a Nm resistance cassette. This strain was created by first PCR amplifying a downstream region and upstream region of the *hsdR* gene and cloning these PCR products into the *XbaI* site of pUCP30T using SLiC (30), then transforming into chemically competent DH5α. This construct was then verified via sequencing using M13 universal primers (Supplemental Table B2). The pUCP30T plasmid (Gm^R) with the *hsdR* upstream/downstream regions was then transformed into an *E. coli* strain harboring pKD46 (Amp^R) which includes genes for lambda red recombinase, the resulting strain was named M2453. A Km/Nm cassette for *hsdR* deletion was then PCR amplified using high-fidelity DNA polymerase and purified. The cassette consisted of the antibiotic resistance gene flanked by regions of homology to the *hsdR* regions cloned into pUCP30T. The cassette was then electroporated into competent M2453 cells (grown in 1 mM Arabinose to induce lambda red recombinase genes) and selected using Km (25 μg mL^{-1}). Cells were then patched on Km (25 μg mL^{-1}), Gm (10 μg mL^{-1}) and Amp (100 μg mL^{-1}). A Km^R, Gm^R, and Amp^S strain was then streak purified and named M2459. The resulting vector was then conjugated into RmP110 as recipient and M2459 as donor. Selection was done in Sm (200 μg mL^{-1}) Nm (200 μg mL^{-1}) plates. Resulting transconjugants were then patched on Gm (10 μg mL^{-1}), Nm (200 μg mL^{-1}) and Sm (200 μg mL^{-1}) plates and a Gm sensitive colony was streak purified (indicating double recombination of cassette with *hsdR* locus). The strain was then verified using diagnostic primers that spanned the *hsdR* upstream/downstream region with the Km/Nm cassette (Supplemental Table B2).

The Nm resistance cassette was then able to be transduced into the *S. meliloti* strains containing various combinations of pSymA and pSymB and selected for Nm resistance. The Nm resistance cassette was then excised by introducing Flp recombinase to flip out the cassette using flanking FRT sites. The resulting strains are RmP4258 (pSymA- pSymB- Δ*hsdR*), RmP4260 (pSymA+ pSymB- Δ*hsdR*), RmP4124 (pSymA- pSymB+ Δ*hsdR*), and RmP4125 (pSymA+ pSymB+ Δ*hsdR*).

2.3.3 Development of *E. coli* ECGE101 Δ*dapA* strain

The *dapA* gene replacement with lambda pir and an erythromycin cassette from strain βDH10B was replicated in Epi300, resulting in a strain containing *trfA* and requirement of DAP supplementation for growth. This is useful because it allows for replication of plasmids with RK2 oriV and makes it a convenient donor in biparental conjugation because the DAP requirement eliminates the need for antibiotic counter-selection. A lambda red recombinase plasmid (pKD46) was electroporated into Epi300, because Epi300 is recA-. The *dapA* region from βDH10B was amplified using the flanking primers DAP1 and DAP2 (Supplemental Table B2). The fragment was electroporated into *E. coli* Epi300 containing pKD46 and transformants were selected on LB with DAP (60 μg mL^{-1}) and erythromycin (200 μg mL^{-1}), and inability to grow in the absence of DAP was confirmed. Such transformants were cured of pKD46 by growing at 37°C and confirming ampicillin (100 μg mL^{-1}) sensitivity.

2.3.4 Vector construction (pAGE/pBGE multi-host shuttle vectors)

Vectors used and created in this study are listed in Supplemental Table B-3. Briefly, pAGE and pBGE vectors were constructed based on the pCC1BAC vector to which elements for replication in yeast were added. This backbone was amplified from plasmid p0521s (created at Venter institute and available on Addgene). This will allow replication and selection in yeast and *E. coli*. Additionally, it is a low copy vector that can be induced to high copy with arabinose. Other components amplified for vector assembly include the antibiotic resistance cassettes for selection in *S. meliloti*, *repA2B2C2* or *repA1B1C1* for replication in *S. meliloti*, origin of transfer (*oriT*) for conjugation, and selective marker for algae (Ntc). The six fragments were PCR amplified and assembled in yeast using a yeast spheroplast transformation method. Next, DNA was isolated from yeast and moved to *E. coli* strain PG5alpha and genotyped using a multiplex PCR screen. Correct vectors were moved from PG5alpha to *E. coli* Epi300 and ECGE101 pTA-Mob. Diagnostic digests were performed to confirm correct assembly of vectors.

2.3.5 Vector stability assay of pAGE2.0

To characterize the ability of the Δ*hsdR* strains to maintain these large pAGE vectors, stability assays were performed to determine how long pAGE2.0 can be maintained in RmP4122 ΔpSymA Δ*hsdR* Nm^s. This was performed in triplicate. Single colonies harboring pAGE2.0 were inoculated into LBmc $FeCl_3$ + $CoCl_2$ with Tc (5 μg mL^{-1}) at 30°C. The next day, 100 μL of culture diluted to 10^{-6} was plated on LBmc $FeCl_3$ + $CoCl_2$ and grown 3 days at 30°C. Cultures were subcultured 1000X in LBmc $FeCl_3$ + $CoCl_2$ without antibiotics and grown overnight. Approximately 10 doublings occurred per day. The next day, cultures were diluted to 10^{-6} and again 100 μL was plated on LBmc $FeCl_3$ + $CoCl_2$. Cultures were subcultured again as before. When visible, 100 colonies were patched onto LBmc $FeCl_3$ + $CoCl_2$ with Tc (5 μg mL^{-1}) and without Tc as a control to ensure colony viability. These plates were incubated at 30°C for 3 days. The number of patched colonies that were able to grow on selective media was then recorded. The experiment was performed for 5 days to assess stability of pAGE2.0.

2.3.6 Electroporation to *S. meliloti*

Preparation of competent *S. meliloti* cells for electroporation: Grow 500 mL of *S. meliloti* overnight in LBmc, 38 μM $FeCl_3$, and streptomycin 100 μg mL^{-1} at 30°C with shaking incubation to an OD_{600} of 2.0. Incubate flask on ice for 10 min then pellet at 6000*g* at 4°C for 10 min. Resuspend cells in 250 mL of sddH_2O by gentile agitation in a water bath, top up volume to 500 mL, then pellet with the same conditions. Repeat this wash step with sddH_2O two additional times, and then repeat once more with the same volume of 10% glycerol. Resuspend the pellet in 3 mL of 10% glycerol, flash freeze 200 μL aliquots and store at -80°C.

Electroporation of *S. meliloti*: Incubate frozen cells on ice until fully thawed (about 15 min). Add 50 ng (in 1 μL) of DNA to 50 μL of competent cells in a 1.5 mL Eppendorf tube on ice, flick to mix, and incubate on ice for 5 min. Add transformation mixture to a 0.1 mm path length cuvette on ice and electroporate at 1.8 kV. Immediately add 1 mL

LBmc 38 μM $FeCl_3$ and recover in a test tube at 30°C with shaking incubation (225 RPM) for 120 min. Spread 500 μL of the transformation mixture (for large plasmids >50 kb) or 10-100 μL (for small plasmids <50 kb) on LBmc plates containing 38 μM $FeCl_3$, 100 μg mL^{-1} streptomycin, and an appropriate concentration of antibiotic selection based on the transformed DNA. Incubate plates at 30°C for 3 days to allow for colony formation.

2.3.7 PEG-mediated transformation to *S. meliloti*

Preparation of competent *S. meliloti* cells for PEG-mediated transformation: *S. meliloti* cells were cultured overnight in LBmc, 38 μM $FeCl_3$, and 100 μg mL^{-1} streptomycin at 30°C with shaking incubation (225 RPM). Upon reaching OD_{600} = 0.4, cells were harvested into sterile 500 mL centrifuge bottles, incubated on ice for 10 min, and pelleted at 4000*g* and 4°C for 10 min. Cells were then resuspended in 100 mL of ice-cold 100 mM $CaCl_2$ by gentle pipetting and incubated on ice for an additional 30 min. Following incubation, cells were pelleted again at 4000*g* and 4°C for 10 min and resuspended in 1.25 mL of ice-cold 100 mM $CaCl_2$ + 15% glycerol. The final resuspension volume was split into 25 μL aliquots, flash frozen using liquid nitrogen, and stored at -80°C for later use.

PEG-mediated transformation of *S. meliloti*: Frozen cells (25 μL aliquots) were incubated on ice until fully thawed. Next, 200 ng (in 3 μL) of supercoiled pAGE DNA and 25 μL of 10% PEG 4000 were then added to the reaction tube and mixed evenly by gentle pipetting. Cells were then incubated on ice for 30 min, transferred to a 40°C water bath for 8 min, and immediately placed back on ice for 10 min. Following, 500 μL of LBmc, 38μM $FeCl_3$ was added to the reaction tube and cells were recovered at 30°C with shaking incubation (225 RPM) for 90 min. Following recovery, 250 μL of the transformation mixture were spread on LBmc plates containing 38 μM $FeCl_3$, 100 μg mL^{-1} streptomycin, and an appropriate concentration of antibiotic selection. Plates were incubated at 30°C for 3 days to allow for colony formation.

2.3.8 Transfer of DNA from *E. coli* to *S. meliloti* via conjugation

Preparation of *S. meliloti* (RmP4122) cells: An overnight culture (OD_{600} = 2.0) grown in LBmc supplemented with $FeCl_3$ (38 µM) and streptomycin (100 µg mL^{-1}) was diluted 20X into the same media to make a 20 mL culture and was grown for 6 hours shaking at 30°C to an OD_{600} of 0.9. The culture was diluted 2000X and grown with shaking at 30°C in 50 mL LBmc supplemented with streptomycin 100 µg mL^{-1} and Fe to OD_{600} of 0.6. The culture was centrifuged for 10 min at 6,000*g* at 4°C and resuspended in 300 µL of LBmc media.

Preparation of *E. coli* (ECGE101 pTA-Mob pAGE1.0) cells: Saturated overnight culture of *E. coli* was diluted 20X into 50 mL LB supplemented with DAP 60 µg mL^{-1}, chloramphenicol 15 µg mL^{-1}, and gentamicin 20 µg mL^{-1} and grown with shaking at 37°C to OD_{600} of 0.6. The culture was centrifuged for 10 min at 6,000*g* at 4°C and resuspended in 300 µL of LBmc media.

Conjugation from *E. coli* to *S. meliloti*: First, 50 µL of *E. coli* cells and 50 µL of *S. meliloti* cells were mixed directly on LBmc plates supplemented with 38µM $FeCl_3$ and DAP 60 µg mL^{-1} and incubated for 180 min at 30°C. Then, 1 mL of LBmc media was added to plates, cells were scraped, and 100 µL (from a dilution series of 10^{-3} to 10^{-9}) was plated on LBmc plates supplemented with Fe, streptomycin 100 µg mL^{-1}, and spectinomycin 200 µg mL^{-1} (Note: plates should be at least 35 mL thick). Plates were incubated at 30°C for 3 days before colonies were counted.

2.3.9 Transfer of DNA from *S. meliloti* to *E. coli* via conjugation

Preparation of *S. meliloti* (RmP4098 pTA-Mob pAGE1.0) cells: Stock culture (OD_{600} = 2.0) was diluted 20X to make 20 mL culture and grown 6 hours shaking at 30°C in LBmc supplemented with streptomycin 100 µg mL^{-1} spectinomycin 200 µg mL^{-1}, gentamicin 40 µg mL^{-1} and Fe to OD_{600} of 0.3. The culture was diluted 500X and grown

with shaking at 30°C in 50 mL LBmc supplemented with streptomycin 100 µg mL^{-1}, spectinomycin 200 µg mL^{-1}, gentamicin 40 µg mL^{-1} to OD_{600} of 0.6. The culture was centrifuged for 10 min at 6,000*g* at 4°C and resuspended in 300 µL of LBmc media.

Preparation of E. coli (Epi300) cells: Saturated overnight culture of *E. coli* was diluted 20X into 50 mL LB and grown with shaking at 37°C to OD_{600} of 0.6. The culture was centrifuged for 10 min at 6,000*g* at 4°C and resuspended in 300 µL of LBmc media.

Conjugation from *S. meliloti* to *E. coli*: First, 50 µL of *E. coli* cells and 50 µL of *S. meliloti* cells were mixed directly on LBmc plates supplemented with Fe and incubated for 180 min at 30°C. Then, 1 mL of LBmc media was added to plates, cells were scraped, and 100 µL (from a dilution series of 10^{-3} to 10^{-9}) was plated on LB plates supplemented with chloramphenicol 15 µg mL^{-1}. Plates were incubated at 37°C for 16 hours before colonies are counted.

2.3.10 Transfer of DNA from *S. meliloti* to *S. cerevisiae* via conjugation

Preparation of *S. meliloti* (RmP4098 pTA-Mob pAGE1.0) cells: Stock culture (OD_{600} = 2.0) was diluted 20x to make 20 mL culture and grown 6 hours shaking at 30°C in LBmc supplemented with streptomycin 100 µg mL^{-1}, spectinomycin 200 µg mL^{-1}, gentamicin 40 µg mL^{-1} and 38µM $FeCl_3$ to OD_{600} of 0.3. The culture was diluted and grown with shaking at 30°C in 120 mL LBmc supplemented with streptomycin 100 µg mL^{-1}, spectinomycin 200 µg mL^{-1}, gentamicin 40 µg mL^{-1}, acetosyringone 100 µg mL^{-1} to OD_{600} of 2.0 (arabinose was added to final concentration of 100 µg mL^{-1} to the growing culture 1 hour before the target OD was reached). The culture was centrifuged for 10 min at 6,000*g* at 4°C and resuspended in 1.5 mL of LBmc media. Note: For simplicity, acetosyringone and arabinose steps can be removed from the final protocol without any substantial decrease in conjugation efficiency.

Preparation of *S. cerevisiae* (VL6-48) cells: *S. cerevisiae* was grown with shaking at 30°C in 100 mL of 2X YPAD media to OD_{600} of 2.5. The culture was centrifuged for 10 min at 5,000*g* and resuspended in 1 mL of H_2O.

Conjugation from *S. meliloti* to *S. cerevisiae*: First, 200 µL of *S. cerevisiae* cells and 250 µL of *S. meliloti* was directly mixed on a 2% -HIS plate supplemented with 10% LBmc, 38 µM $FeCl_3$ and acetosyringone 100 µg mL^{-1} (Note: plates were dried out in the hood for 1 hour prior to conjugation). Then plates were incubated for 180 min at 30°C. Then, 2 mL of sddH_20 was added to plates and cells were scraped. Next, 100 µL of the scraped cells was plated on 2% -HIS plates supplemented with ampicillin 100 µg mL^{-1}. Plates were incubated at 30°C where colonies start to appear after 2-3 and colonies are counted after 5 days. Note: For simplicity, acetosyringone steps can be removed from the final protocol without any substantial decrease in conjugation efficiency.

2.3.11 Transfer of DNA from *S. meliloti* to *P. tricornutum* via conjugation

Preparation of *P. tricornutum* cells: First, 250 µL of liquid grown culture was adjusted to 1.0 x 10^8 cells mL^{-1} using counts from a hemocytometer, was plated on ½L1 1% agar plates and grown for 4 days. Then, 1 mL of L1 media was added to the plate and cells were scraped, counted using a hemocytometer, and adjusted to a concentration of 1 x 10^9 cells mL^{-1}.

Preparation of *S. meliloti* (RmP4098 pTA-Mob pAGE1.0) cells: Stock culture (OD_{600} = 2.0) was diluted 20X to make 20 mL culture and grown 6 hours shaking at 30°C in LBmc supplemented with spectinomycin 200 µg mL^{-1}, gentamicin 40 µg mL^{-1} and Fe to OD_{600} of 0.3. The culture was diluted 25X and grown for 12 hours with shaking at 30°C in 50 mL LBmc supplemented with spectinomycin 200 µg mL^{-1}, gentamicin 40 µg mL^{-1} to OD_{600} of 0.6. The culture was centrifuged for 10 min at 5,000*g* at 4°C and resuspended in 500 µL of LBmc media.

Conjugation from *S. meliloti* to *P. tricornutum*: First, 200 µL of *P. tricornutum* cells and 200 µL of *S. meliloti* cells were mixed directly on ½L1 10% LBmc 1% agar plates (Note: plates are dried in the biosafety cabinet for one hour before conjugation) and incubated for 180 min at 30°C in the dark, then moved to 18°C in the light and grown for 2 days. After two days, 2 mL of L1 media was added to plates, cells were scraped, and 100 µL (5%) was plated on ¼L1 1% agar plates supplemented with nourseothricin 100 µg mL^{-1}, and ampicillin 100 µg mL^{-1} (Note: plates should be at least 35 mL thick). Plates were incubated at 18°C in the light/dark cycle and colonies start to appear after 7 days and are allowed to develop to 14 days before colonies are counted.

2.3.12 Transfer of DNA from *S. meliloti* to *P. tricornutum* via conjugation in a 96-well plate

Preparation of *P. tricornutum* cells: First, 200 µL of liquid grown culture was diluted using counts from a hemocytometer, and grown in ½L1 media for 4 days. Cell counts from a hemocytometer were used and culture was pelleted at 4000*g* 10 min 4°C, and adjusted to a concentration of 1 x 10^9 cells mL^{-1}.

Preparation of *S. meliloti* (RmP4098 pTA-Mob pAGE1.0) cells: Stock culture (OD_{600} = 2.0) was diluted 20x to make 20 mL culture and grown 6 hours shaking at 30°C in LBmc supplemented with spectinomycin 200 µg mL^{-1}, gentamicin 40 µg mL^{-1} and Fe to OD_{600} of 0.3. The culture was diluted 25X and grown for 12 hours with shaking at 30°C in 50 mL LBmc supplemented with spectinomycin 200 µg mL^{-1}, gentamicin 40 µg mL^{-1} to OD_{600} of 0.6. The culture was centrifuged for 10 min at 5,000*g* at 4°C and resuspended in 500 µL of LBmc media.

Conjugation from *S. meliloti* to *P. tricornutum*: First, 5 µL of *P. tricornutum* cells and 5 µL of *S. meliloti* cells were mixed together in a 96-well plate. The mixture (10 µL) was transferred to a 96-well plate containing 200 µL of ½L1 10% LBmc 1% agar (note: plates are dried in the biosafety cabinet for one hour before conjugation). This conjugation plate was incubated for 180 mins at 30°C in the dark, then moved to 18°C in the light and

grown for 2 days. After two days, 100 µL of L1 media was added to wells and cells were scraped (X2), and 10 µL (5%) was plated on ¼L1 1% agar supplemented with nourseothricin 100 µg mL^{-1}, and ampicillin 100 µg mL^{-1} in a 96-well plate. Plates were incubated at 25°C in the light/dark cycle for 24 hours and then 18°C in the light/dark cycle for an additional 24 hours. Colonies start to appear after 7 days and are allowed to develop up to 14 days before colonies are counted.

2.3.13 Plasmid DNA isolation

Plasmid DNA <60 kb was isolated from *E. coli* using the BioBasic EZ-10 miniprep kit and from all other species using the modified alkaline lysis protocol described below. Plasmid DNA >60 kb was isolated from all species using the modified alkaline lysis protocol. Steps 1–3 are variable depending on the species, while steps 4–10 are common for all species. Steps 1–3 for *E. coli* and *S. meliloti*. (1) Five mL cultures were grown to saturation overnight. (2) Cells were pelleted at 5000*g* for 10 min at 4°C, and the supernatant was discarded. (3) Cells were resuspended in 250 µL of resuspension buffer (which contained 240 mL P1 (Qiagen), 5 mL of 1.4 M β-Mercaptoethanol and 5 mL Zymolyase solution (200 mg Zymolyase 20T (USB), 9 mL H_2O, 1 mL 1 M Tris pH 7.5, 10 mL 50% glycerol, stored at 20°C). Steps 1–3 for *S. cerevisiae*. (1) Five mL of culture was grown to saturation. (2) Cells were pelleted at 5000*g* for 10 min at 4°C, and the supernatant was discarded. (3) Cells were resuspended in 250 mL of resuspension buffer (as described above) and incubated at 37°C for 60 min. Steps 1–3 for *P. tricornutum*. (1) Five mL cultures were harvested during exponential growth phase. (2) Cells were pelleted at 4,000*g* for 10 min at 4°C, and the supernatant was discarded. (3) Cells were resuspended in 250 mL of resuspension buffer, which contained 235 mL P1 (Qiagen), 5 mL hemicellulase 100 mg mL^{-1}, 5 mL of lysozyme 25 mg mL^{-1}, and 5 mL Zymolyase solution and then cells were incubated at 37°C for 30 min. Steps 4–10 are common for all species. (4) 250 µL of lysis buffer P2 (Qiagen) was added and samples were inverted 5–10 times to mix. (5) 250 mL of neutralization buffer P3 was added and samples were inverted 5–10 times to mix. (6) Then samples were spun down at 16,000*g*, 10 min at 4°C (7) Supernatant was transferred to a clean tube and 750 µL ice-cold isopropanol was

added and the samples were mixed by inversion and spun down at 16,000*g*, 10 min at 4°C (8) Next the supernatant was removed and 750 µL ice-cold 70% EtOH was added and samples were mixed by inversion and spun down at 16,000*g*, 5 min. (9) Next the supernatant was discarded, pellets were briefly dried and resuspended in 50 µL of TE buffer. (10) After that the samples were kept at 37°C for 30–60 min to dissolve.

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Chapter 3

3 Conjugation-based genome engineering in *Deinococcus radiodurans*

The work presented in this chapter is reproduced (with permission – Appendix A) from:

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3.1 Introduction

Deinococcus radiodurans is a polyextremophile bacterium that is well-known for its resistance to DNA-damaging agents including ionizing and UV radiation (1), desiccation (2), and the vacuum of space (3). These characteristics make *D. radiodurans* an attractive chassis for biotechnology as it can withstand the physiochemical stress of industrial processes that traditional model organisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*, cannot. Currently, biotechnological applications of this organism include bioremediation (4), nanomaterial biosynthesis (5), and unique pigment production (6).

The expanding genetic toolkit for *D. radiodurans* includes shuttle plasmids (7), selective markers (8), characterized promoters for inducible gene expression (9), and strategies for generating gene deletions using a Cre-lox system (10). The genome of *D. radiodurans* R1 was first sequenced in 1999 by White *et al*. revealing a G+C-rich (67%), multipartite genome composed of two chromosomes (2.6 Mb and 412 kb), a megaplasmid (MP1, 177 kb), and a small plasmid (CP1, 46 kb) (11). Recent PacBio sequencing of this strain identified both large insertions and single nucleotide polymorphisms (SNPs) when mapped to the original sequence (12). Although *D. radiodurans* was one of the first bacteria to be sequenced, tools to enable whole genome engineering such as those used for engineering *Mycoplasma mycoides* (13) and *E. coli* (14) have yet to be developed.

These large-scale genetic manipulations typically involve cloning of synthetic DNA fragments in host organisms, such as *E. coli* or yeast, followed by transfer to destination cells via transplantation (15), bacterial conjugation (16), or electroporation (17). In *D. radiodurans*, exogenous DNA is most commonly introduced by natural or chemical transformation (18). However, these methods often require large amounts of DNA, likely due to active restriction-modification (R-M) systems known to digest exogenous DNA, and have not been demonstrated to transfer large genomic regions or whole chromosomes. Therefore, toward the goal of whole genome engineering in this species, methods for DNA delivery to *D. radiodurans* must first be improved.

Bacterial conjugation is a mechanism of horizontal gene transfer between cells in direct contact and has been demonstrated between Gram-negative and Gram-positive bacteria. In previous studies *E. coli* was shown to conjugate to several Gram-positive bacteria using an IncP RK2 plasmid including *Enterococcus, Streptococcus, Bacillus, Listeria* and *Staphylococcus* (19), *Streptomyces* (20), and more recently, plasmid transfer from *E. coli* to *Bifidobacterium* (21) and *Lactobacillus* (22) was demonstrated. Conjugation has not yet been demonstrated to *D. radiodurans* but it is an attractive solution and established method for the transfer of large DNA constructs between bacteria and from bacteria to eukaryotes (23–25), the evasion of native restriction systems (26), and the propagation of plasmids through microbial communities and biofilms (27–29).

Here, as the first step toward genome-scale engineering in *D. radiodurans*, we have developed conjugation as a method to transfer replicating and nonreplicating plasmids from *E. coli* directly to *D. radiodurans*. We demonstrated a conjugation-based method for creating targeted gene deletions *in vivo*, replacing four R-M genes with antibiotic resistance markers. Finally, we cloned the whole 178 kb MP1 megaplasmid from the *D. radiodurans* genome in *E. coli* using conjugation-based plasmid integration.

3.2 Results and Discussion

3.2.1 Conjugation as a new DNA delivery method to *D. radiodurans*

To test whether existing methods of DNA transfer to *D. radiodurans* were amenable to large (>20 kb) plasmids, we performed $CaCl_2$-based transformation using a 3 kb linear fragment, a 6 kb plasmid, and a 22 kb plasmid. As shown in Table 2-1, transformation efficiency of the linear fragment and small plasmid were 7.5×10^5 and 2.7×10^1 CFU/μg of DNA, while no transformants were obtained for the larger plasmid. This result demonstrates the necessity of developing conjugation-based methods for the transfer of large DNA constructs.

Therefore, we developed conjugation as a direct method of DNA transfer from *E. coli* to *D. radiodurans* (Figure 3-1). To achieve this, we first constructed a replicating cargo plasmid, pDEINO1, by assembling a codon-optimized chloramphenicol acetyltransferase (*cat*) gene and an origin of replication (30) for *D. radiodurans* into the pAGE3.0 (31) multihost shuttle (MHS) plasmid (Figure 3-2A). We used *E. coli* Δ*dapA* as the donor strain (31) to allow easy counterselection and wild-type *D. radiodurans* as the recipient. The donor strain harbored the IncP RK2 conjugative plasmid pTA-Mob (32), which encodes all the genes required to transfer the cargo plasmid, and pDEINO1. The mean conjugation frequency of pDEINO1 from *E. coli* to *D. radiodurans* was 1.12×10^{-5} transconjugants per recipient (Figure 3-2B). No transconjugant colonies formed when *E. coli* harboring pDEINO1, but lacking pTA-Mob, was used as a donor or when the recipient was plated with no *E. coli* donor.

Additional experiments were performed to verify that conjugation was the mechanism of plasmid transfer (Figure 3-3). No *D. radiodurans* transconjugants were observed using an *E. coli* donor harboring a pTA-Mob Δ*traI* plasmid, where *traI* is an essential conjugative gene encoding for relaxase, or when 1 μg of pDEINO1 DNA was directly mixed with recipient cells. Furthermore, the addition of DNase throughout the conjugative protocol had no impact on the number of transconjugant colonies obtained when an *E. coli* donor harboring pTA-Mob and pDEINO1 was used.

Table 3-1. Transformation efficiency of DNA constructs into *D. radiodurans* $CaCl_2$-competent cells. Transformation efficiency of DNA constructs including a linear cassette from pDEINO10 (~3.3 kb, 69 ng total), the pRAD1 plasmid (~6.2 kb, 1.12 µg total), and the pDEINO1 plasmid (~22 kb, 460 ng total) into *D. radiodurans* $CaCl_2$-competent cells. The pDEINO1 and no DNA control transformations were plated on both neomycin and chloramphenicol selection, while the linear cassette and pRAD1 transformations were plated on neomycin (Nm) or chloramphenicol (Cm), respectively. TNTC, too numerous to count; N/A, not applicable.

Transformed DNA	Dilution	Selection (µg mL^{-1})	Colony Count	Transformation Efficiency (CFU µg DNA^{-1})
Linear cassette	10^0	Nm 5	TNTC	N/A
	10^{-1}	Nm 5	1485	7.5×10^5
pRAD1	10^0	Cm 3	15	2.7×10^1
	10^{-1}	Cm 3	1	3.1×10^1
pDEINO1	10^0	Cm 3	0	0
	10^{-1}	Cm 3	0	0
	10^0	Nm 5	0	0
	10^{-1}	Nm 5	0	0
No DNA	10^0	Cm 3	0	0
	10^{-1}	Cm 3	0	0
	10^0	Nm 5	0	0
	10^{-1}	Nm 5	0	0

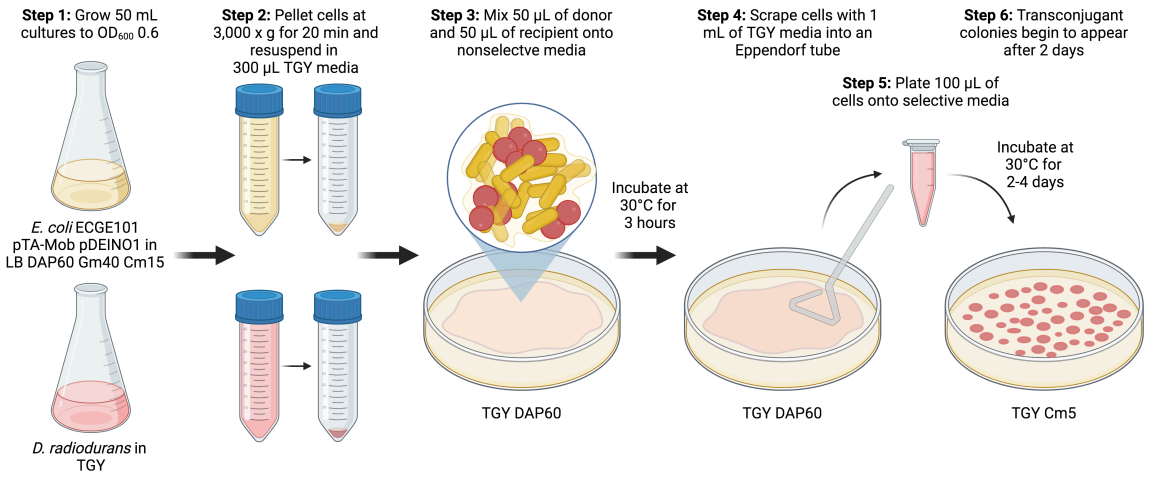

Figure 3-1. Flowchart of the *E. coli* to *D. radiodurans* conjugation protocol. Antibiotic concentrations are reported in µg mL^{-1}. Created with BioRender.com.

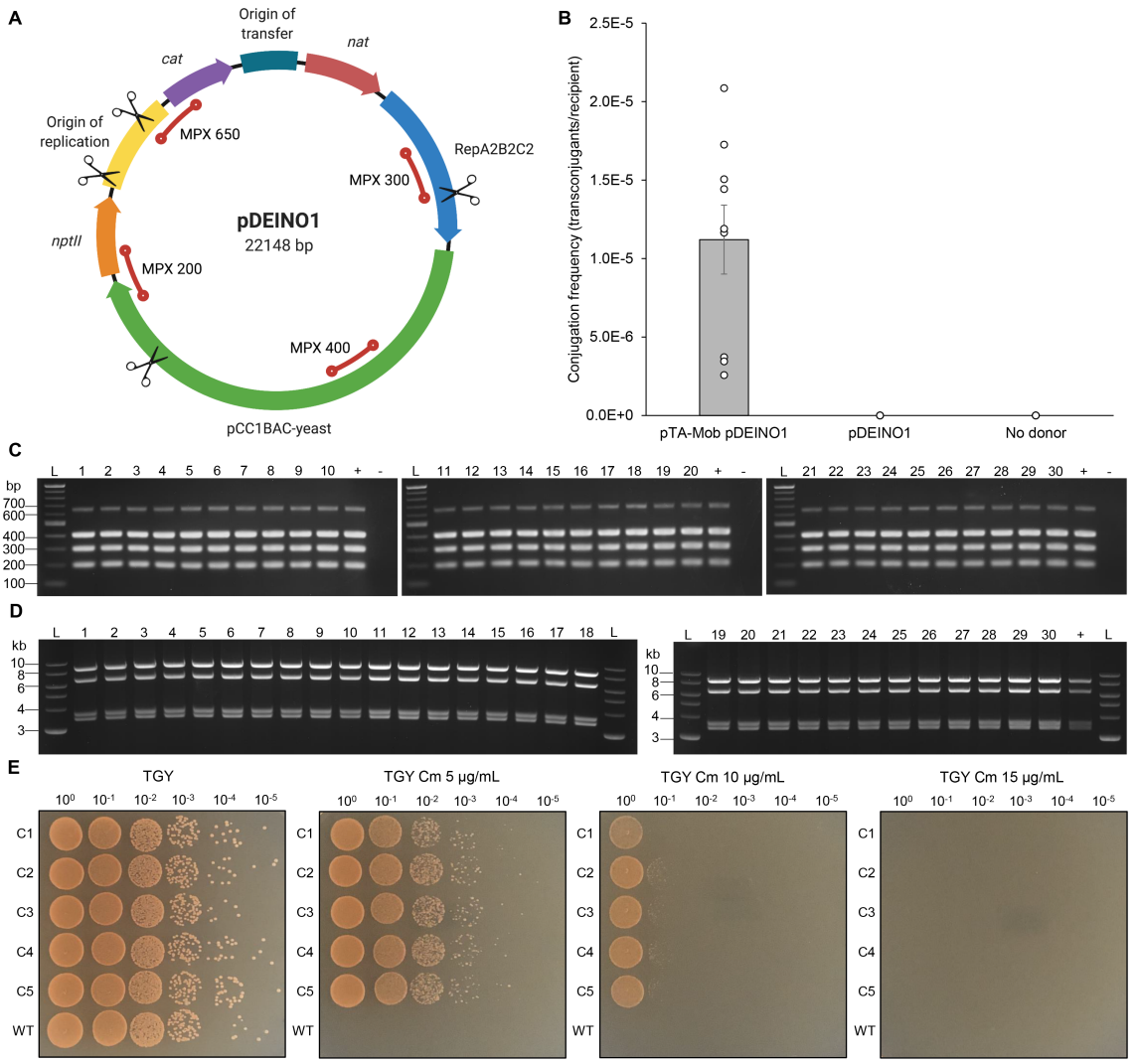

Figure 3-2. Conjugation of pDEINO1 from *E. coli* to *D. radiodurans*. (A) Schematic of the MHS plasmid pDEINO1: *cat*, chloramphenicol resistance gene; *nat*, *N*-acetyltransferase; *nptII*, neomycin phosphotransferase II; RepA2B2C2, *Sinorhizobium meliloti* origin of replication; pCC1BAC-yeast, backbone for selection and replication in yeast and *E. coli*. Red lines indicate the location and size (bp) of multiplex amplicons, and scissors indicate *Eco*RI cut sites. Created with BioRender.com. (B) Conjugation frequency of pDEINO1 to *D. radiodurans* from *E. coli* donors harboring pTA-Mob and pDEINO1, only pDEINO1, or without a donor. Data are shown as a bar graph with points representing individual technical replicates. Error bars represent standard error of the mean. (C,D) Agarose gel of multiplex PCR products and restriction digest of 30 pDEINO1 plasmids isolated from *D. radiodurans* transconjugants, following transformation and plasmid induction in *E. coli*. L, 2-log ladder; +, original pDEINO1 plasmid; −, water. (C) Expected multiplex amplicon sizes are 200, 300, 400, and 650 bp. (D) Expected *Eco*RI-HF restriction digest band sizes are 3149, 3450, 6548, and 8474 bp. (E) Phenotypic screening for chloramphenicol sensitivity of *D. radiodurans* transconjugants harboring pDEINO1 relative to wild-type *D. radiodurans* by serial

dilution spot plates on TGY media and TGY media supplemented with increasing concentration of chloramphenicol. C1–C5, *D. radiodurans* transconjugant colonies. WT, wild-type *D. radiodurans.*

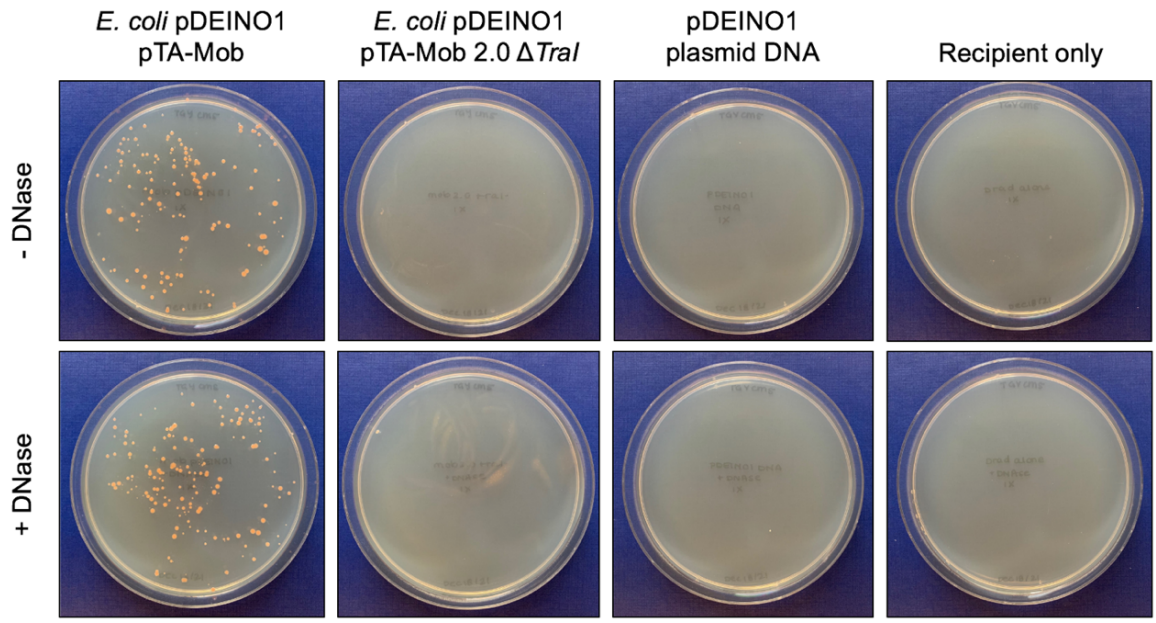

Figure 3-3. Conjugation with DNase treatment, a *traI* mutant donor or pDEINO1 DNA. Conjugation of pDEINO1 to *D. radiodurans* using an *E. coli* donor harbouring pDEINO1 and pTA-Mob, an *E. coli* donor harboring pDEINO1 and pTA-Mob 2.0 Δ*traI,* 1 µg of pDEINO1 DNA directly mixed with recipient cells, or recipient only. All conditions were performed with (+) and without (-) the addition of DNase and were plated on TGY media supplemented with Cm 5 µg mL^{-1}. The number of colonies obtained using *E. coli* pDEINO1 pTA-Mob with (+) and without (-) DNase was 164 and 149, respectively.

To confirm that the *D. radiodurans* transconjugants harbored pDEINO1, we isolated plasmids from 30 individual transconjugant colonies. The DNA was transformed into *E. coli* Epi300, and plasmids from 30 single colonies were induced to high copy number with arabinose. The plasmids were analyzed by multiplex PCR and diagnostic restriction digest (Figure 3-2C,D), both of which showed the expected banding patterns following gel electrophoresis. Whole-plasmid sequencing analysis from two transconjugant colonies showed no mutations when compared to the original pDEINO1 sequence. These results demonstrate that replicating MHS plasmids can be successfully conjugated from *E. coli* to *D. radiodurans* with no gross rearrangements or point mutations.

The codon-optimized *cat* gene was an effective plasmid-based marker for selection of *D. radiodurans* transconjugants. To determine the sensitivity of *D. radiodurans* carrying pDEINO1 to chloramphenicol, five transconjugant colonies were spot-plated alongside the wild-type strain on varying concentrations of chloramphenicol (Figure 3-2E). We found that an antibiotic concentration of 5 μg mL^{-1} inhibited the growth of wild-type *D. radiodurans*, while having only a small negative effect on transconjugants; however, any additional increase in chloramphenicol concentration significantly impacted transconjugant growth. Additionally, we verified that the *E. coli cat* gene present on pDEINO1 was not able to provide resistance for *D. radiodurans* when introduced on pDEINO6 without the *D. radiodurans cat* gene (Supplemental Figure C-1).

Plasmid curing is beneficial for genome engineering strategies dependent on the recycling of plasmids or that only require plasmids temporarily for expression of an endonuclease (*e.g.*, Cas9). To demonstrate that the strains could be cured of the plasmid, we performed a plasmid stability assay on one transconjugant colony grown without selection in liquid media for approximately 40 generations. We showed that on average 44% of cells lost the pDEINO1 plasmid every 13 generations when grown without selection (Table 3-2). Our results are the first demonstration harnessing conjugative machinery for delivery of plasmids in *D. radiodurans*.

Table 3-2. Plasmid stability assay of pDEINO1 in *D. radiodurans*. The plasmid stability assay was performed on a single *D. radiodurans* transconjugant harboring pDEINO1 over 40 generations. The nonselective media was TGY, while selective media was TGY Cm 5 μg mL^{-1}.

Day Number	Colonies on Nonselective Media (x 10^4)	Colonies on Selective Media (x 10^3)	Surviving Colonies Restruck on Selective Media
1	116	282	71
2	105	109	32
3	140	55	14
4	106	11	6

3.2.2 Incorporation and analysis of selectable markers

To design plasmid-based systems utilizing this new conjugative delivery method, we individually installed three additional antibiotic markers: *tetR/A*, *aadA1*, or *aacC1*, conferring resistance to tetracycline, streptomycin, and gentamicin, respectively, on replicating plasmids also containing a codon-optimized *cat* gene (Figure 3-4A). These plasmids, called pDEINO3, pDEINO4, and pDEINO5, as well as pDEINO1 were conjugated into *D. radiodurans*. Transconjugant colonies were selected on media supplemented with chloramphenicol and were subsequently spotted onto various antibiotics. As shown in Figure 3-4B, all strains harboring the MHS plasmids conferred resistance to chloramphenicol and their respective antibiotic, while exhibiting sensitivity to the other three antibiotic supplements. In addition to showing chloramphenicol resistance, the pDEINO1 transconjugant also showed resistance to neomycin and kanamycin since *nptII* confers resistance to both antibiotics (Figure 3-4B, Supplemental Figure C-2). Notably, chloramphenicol at a concentration of 5 μg mL^{-1} inhibited cell growth approximately 10-fold compared to other antibiotics. However, as shown in Supplemental Figure C-1, this growth inhibition can be overcome by lowering the concentration of chloramphenicol to 3 μg mL^{-1}. Furthermore, we observed that supplementing gentamicin at a concentration as little as 2 μg mL^{-1} sufficiently inhibited wild-type *D. radiodurans*, although there was some breakthrough at the lowest spot plate dilution.

3.2.3 Conjugation-based gene deletions using nonreplicating plasmids

Gene deletions have previously been demonstrated in *D. radiodurans* using targeted integration of linear DNA fragments or plasmids containing regions homologous to the genome flanking a selective marker (33). This strategy has been used to introduce biosynthetic pathways into *D. radiodurans* for applications including pollutant degradation or detoxification of mercury and toluene (4,34). More recently, multiple knockouts of genes replaced by selective markers have been demonstrated by

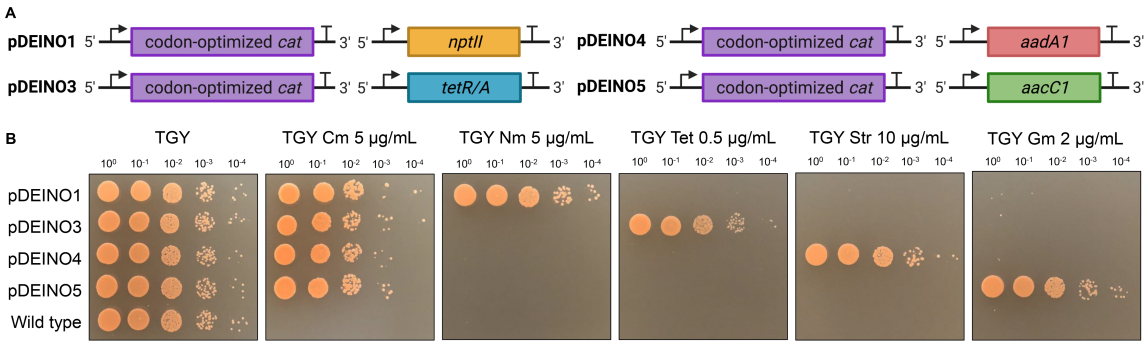

Figure 3-4. Effect of plasmid-based selective markers on antibiotic resistance in *D. radiodurans*. (A) Diagram of pDEINO1, pDEINO3, pDEINO4, and pDEINO5 selective marker cassettes containing a *cat* gene codon-optimized for *D. radiodurans* coupled with *nptII*, *tetR/A*, *aadA1*, or *aacC1*, respectively. Created with BioRender.com. (B) Serial dilutions of *D. radiodurans* transconjugants harboring pDEINO1, pDEINO3, pDEINO4, and pDEINO5 alongside wild-type *D. radiodurans* spot plated on TGY media and TGY media supplemented with chloramphenicol (Cm), neomycin (Nm), tetracycline (Tet), streptomycin (Str), and gentamicin (Gm).

recombination of nonreplicating plasmid in *D. radiodurans* (8), as well as a Cre-lox recombination system (10).

With an efficient plasmid delivery method and a collection of selective markers, we sought to develop a plasmid-based protocol for generating gene deletions in *D. radiodurans.* We chose four R-M genes as targets, some of which have been deleted or disrupted in *D. radiodurans* in previous studies (35–37). These genes include *Mrr* and ORF2230 located on chromosome 1, ORF14075 on chromosome 2, and ORF15360 on the MP1 megaplasmid (Figure 3-5A). Prior to plasmid construction we sequenced the *D. radiodurans* R1 strain using the Oxford Nanopore MinION platform to ensure accurate homology regions were incorporated into the plasmid designs. Sequencing revealed the genome size of chromosome 1, chromosome 2, MP1 and CP1 to be 2.6 Mb, 412 kb, 178 kb and 45 kb, respectively. Interestingly, our results did not detect the large insertions as reported in the PacBio sequencing results (12), and as such the genome size more closely resembles the original sequence published by White *et al*. (1999) (2.6 Mb, 412 kb, 177 kb, 46 kb) compared to the PacBio sequence (2.6 Mb, 433 kb, 203 kb, 62 kb) (11).

The four nonreplicating plasmids, pDEINO7–10, were constructed with a backbone for replication and selection in *E. coli* and *S. cerevisiae*, an *oriT* for conjugative transfer, and a deletion cassette containing a selective marker–neomycin or tetracycline–between 1 kb regions of homology flanking the gene targeted for deletion (Figure 3-5B). Following conjugative delivery, transconjugants were selected with the respective antibiotic, DNA was isolated from *D. radiodurans* and screened for genomic gene deletions using multiplex PCR. Two transconjugants were screened for each deletion event, and the PCR showed amplification of only the selectable marker, indicating successful integration of the selectable marker, deletion of the targeted gene, and loss of the plasmid backbone (Figure 3-5C). To show that this system could also be used to generate multiple deletions in the same strain, we performed conjugation using an ORF15360 deletion transconjugant as the recipient and an *E. coli* donor harboring pDEINO7 targeting ORF14075. Transconjugants were selected on media supplemented with both neomycin and tetracycline, and their DNA was screened using the same multiplex PCRs for each single deletion. Analysis of two clones confirmed that both

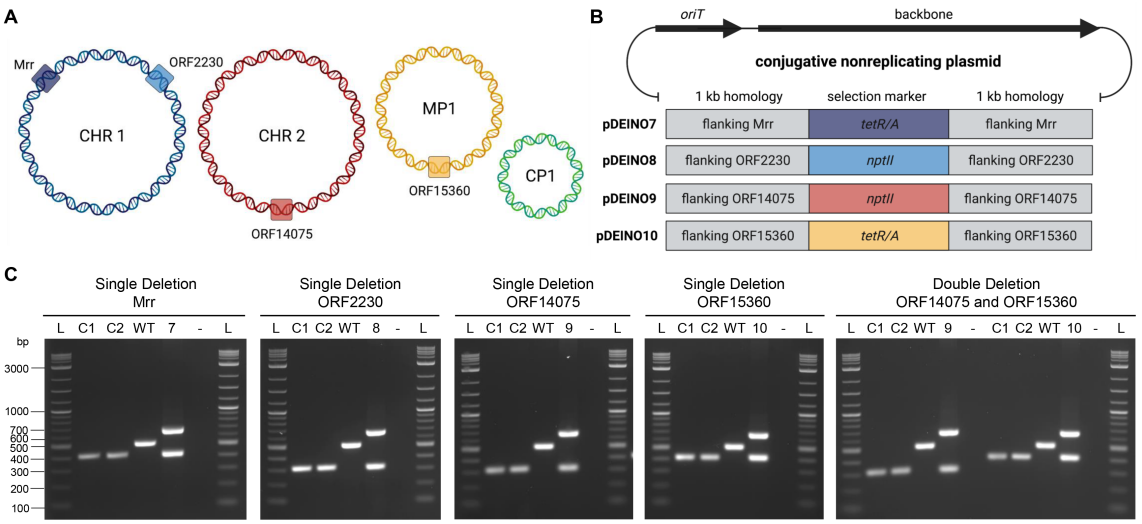

Figure 3-5. Gene deletions in *D. radiodurans* using recombination of conjugative nonreplicating plasmids. (A) Diagram of *D. radiodurans* genome with four R-M gene targets indicated as colored boxes. (B) Conjugative nonreplicating plasmid map illustrating the components of pDEINO7–10 deletion cassettes, used to generate the *D. radiodurans* deletion strains. Graphics A and B created with BioRender.com. (C) Agarose gels showing multiplex PCR analysis of two *D. radiodurans* transconjugant colonies for each R-M gene knockout (C1, C2). Controls included wild-type *D. radiodurans* genomic DNA (WT), the original pDEINO7–10 plasmids extracted from *E. coli* (7, 8, 9, 10), and water (−). If present, amplicon sizes are neomycin 311 bp, tetracycline 409 bp, wild-type R-M genes ~500 bp, and the nonreplicating plasmid backbone 645 bp. L, 2-log ladder.

ORF15360 and ORF14075 genes were replaced with the tetracycline and neomycin markers, respectively, and the plasmid backbone was lost (Figure 3-5C).

The multicopy genome of *D. radiodurans* can make genetic modification more difficult as integrated sequences are often heterozygous and can be deleted by intrachromosomal recombination events (38,39). Using our method, and after restreaking transconjugants twice on selective plates, no amplification of the target R-M genes was observed in the knockout strains following the multiplex PCR (Figure 3-5C). To confirm homogeneous knockout of these genes, we performed whole-genome Nanopore sequencing of the double knockout *D. radiodurans* strain and aligned the reads to the wild type sequence (Figure 3-6). There was no coverage at the sequence of ORF14075 on chromosome 2 or ORF15360 on the MP1 plasmid, confirming that we have generated a strain with multiple homogeneous gene knockouts. Successful deletion of multiple genes proves that this technique could be implemented to sequentially delete genes involved in recognizing and digesting foreign or synthetic DNA in the cell in order to develop a restriction-free *D. radiodurans* strain.

3.2.4 Conjugation-based cloning of the MP1 megaplasmid

To engineer *D. radiodurans* as an industrial chassis, streamlined strains, such as those developed for *Mycoplasma mycoides* JCVI-syn3.0 (40), may be developed by building synthetic genomes. Creating a synthetic or minimal *D. radiodurans* cell will require the use of intermediate host organisms, such as *E. coli*, yeast, or *Sinorhizobium meliloti* to house the DNA for genetic modification and cloning (31,41,42). To this end, we aimed to demonstrate that large DNA fragments from the *D. radiodurans* genome could be hosted in *E. coli* by cloning the native MP1 megaplasmid, 178 kb in length with a notable G+C content of ~62%.

We built a nonreplicating plasmid called pDEINO2 with a 1 kb region of homology to the middle of the *McrC* gene on the MP1 megaplasmid to facilitate recombination and integration of the entire plasmid (Figure 3-7A) and delivered it to *D.*

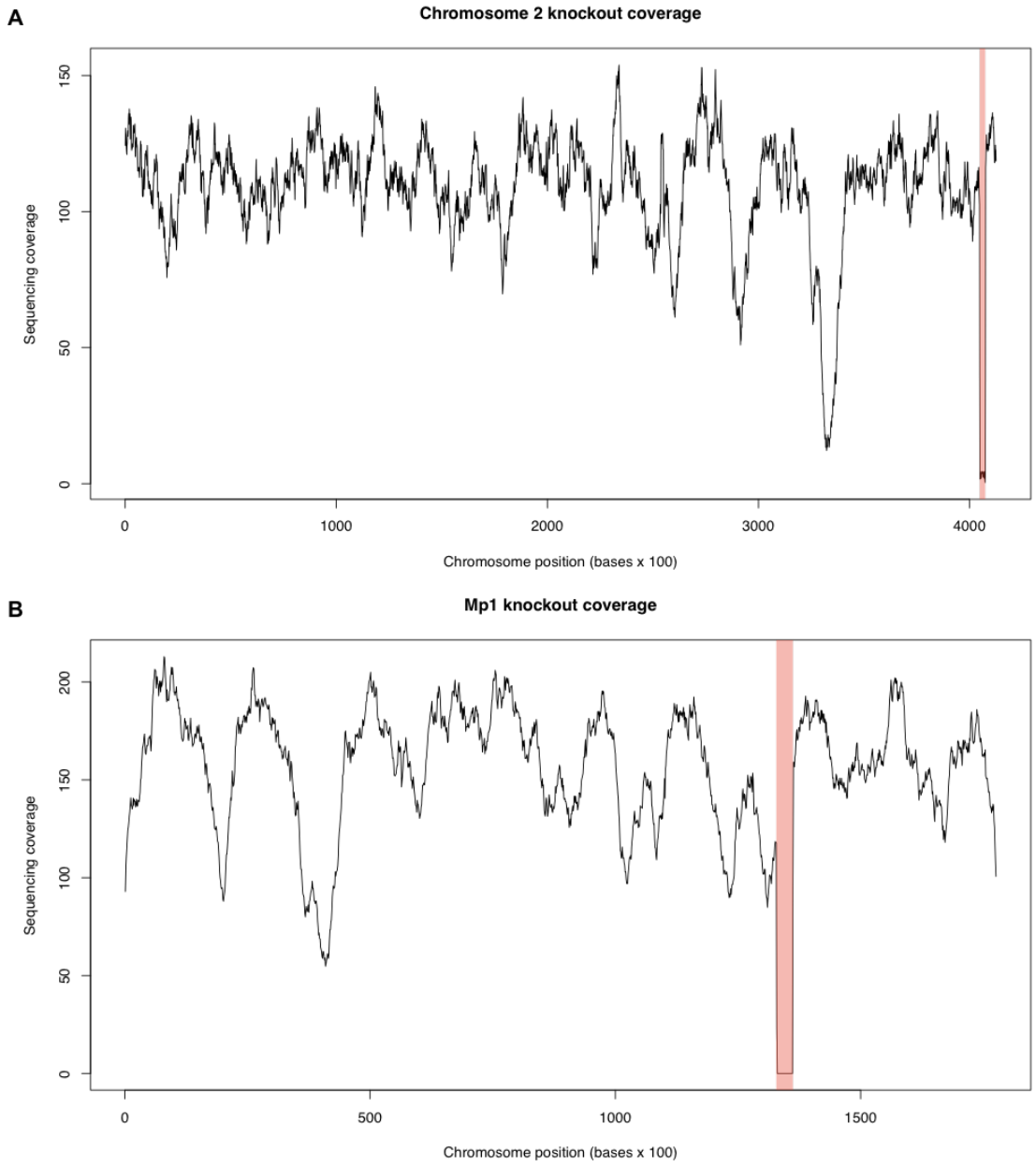

Figure 3-6. Alignment of *D. radiodurans* double knockout sequencing reads to the wild-type genome. The *D. radiodurans* double knockout strain (Δ14075 Δ15360) was sequenced using the RBK004 kit on an Oxford Nanopore MinIon R9.4.1 flow cell according to the manufacturer's protocol. Reads were basecalled using Guppy v5.015 in high-accuracy mode. Reads were aligned against the wild-type genome (ENA: SAMEA9996585) using minimap2 (A) for chromosome 2 and (B) for the MP1 plasmid. Coverage was calculated using mosdepth in 100 base windows. The regions expected to have zero coverage due to gene knockouts are highlighted in red.

radiodurans via conjugation (Figure 3-7B). We extracted DNA from transconjugants selected with chloramphenicol and performed a multiplex PCR confirming integration of pDEINO2 (Figure 3-7C, Supplemental Figure C-3). Extracted DNA was then transformed into *E. coli*, individual colonies were induced to high copy number, and isolated DNA was screened to verify cloning and propagation of the MP1 megaplasmid without compromising genomic integrity. We confirmed integration of pDEINO2 at the *McrC* locus using two diagnostic restriction digests (Figure 3-7D) and multiplex PCR amplifying regions of pDEINO2 and the *D. radiodurans* MP1 megaplasmid (Figure 3-7E). In particular, the presence of the 7733 bp band and 8112 bp band in the MfeI-HF and NheI-HF digests, respectively, was a strong indicator of plasmid integration at the *McrC* locus, as these are unique band sizes created by the integration event that would otherwise not be present in the wild-type MP1 plasmid. Finally, we extracted total DNA from one *E. coli* clone harboring the pDEINO2-MP1 plasmid and sequenced it using the Oxford Nanopore MinION platform. The cloned MP1 plasmid sequence was assembled and confirmed that pDEINO2 successfully integrated at the intended target site and the final plasmid size was ∼190 kb. These results indicate that large regions of the *D. radiodurans* genome, including entire replicons, can be effectively cloned and propagated in *E. coli*. Additionally, isolation of the *D. radiodurans* megaplasmid has intriguing applications. Megaplasmids are large, extrachromosomal replicons that are commonly acquired through horizontal gene transfer. These replicons often provide beneficial, niche-specific traits to the host, such as antimicrobial resistance, symbiosis, or metabolic pathways (43).

In summary, we have demonstrated conjugation from *E. coli* as a DNA delivery method for *D. radiodurans* and created several conjugation-based tools for *in vivo* genome engineering including strategies for gene deletions and whole chromosome cloning. We generated four single and one double R-M gene knockout in *D. radiodurans*, demonstrating this conjugation-based method as a promising strategy for generating genomic deletions or integrating genes and biosynthetic pathways. Furthermore, we showed that *E. coli* can be a suitable host for maintaining large, high G+C content plasmids as demonstrated by cloning the MP1 megaplasmid from *D. radiodurans*. We anticipate that the genetic tools described here will advance the engineering process,

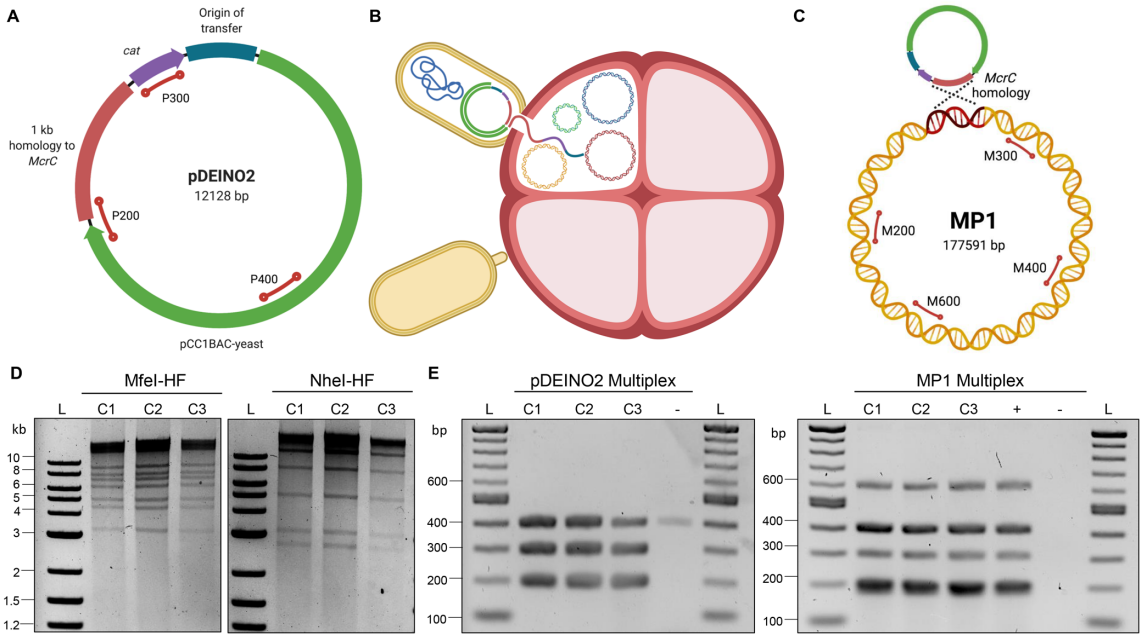

Figure 3-7. Cloning of *D. radiodurans* MP1 megaplasmid in *E. coli*. (A) Schematic of the pDEINO2 nonreplicating plasmid. Multiplex amplicon locations and sizes (bp) are indicated as red lines. (B,C) Diagrams illustrating conjugation of the pDEINO2 plasmid from *E. coli* to *D. radiodurans* and integration into the MP1 plasmid through recombination at the *McrC* gene. Multiplex amplicon locations and sizes are indicated as red lines. Graphics A–C created with BioRender.com. (D) Agarose gel showing MfeI-HF and NheI-HF restriction digests of cloned pDEINO2-MP1 plasmids recovered from three *E. coli* clones following induction. Expected visible band sizes (<10 kb) are 325, 3186, 4338, 4929, 6170, 7049, 7733, and 8948 bp for MfeI-HF and 2701, 3139, 5070, and 8117 bp for NheI-HF. (E) Agarose gel showing multiplex PCR of three cloned pDEINO2-MP1 plasmids recovered from *E. coli*. The pDEINO2 amplicons are 200, 300, and 400 bp and the MP1 amplicons are 200, 300, 400, and 600 bp. L, 2-log ladder; +, wild-type *D. radiodurans* genomic DNA; −, water. Note: Nonspecific amplification of a weak band can be seen around 400 bp in the negative control of the pDEINO2 multiplex.

particularly for modification of whole genomes, and allow for the development of *D. radiodurans* strains controlled by designer synthetic genomes for industrial applications, as well as foundational biology studies.

3.3 Methods

3.3.1 Microbial strains and growth conditions

Deinococcus radiodurans R1 was grown at 30°C in TGY medium (5 g L^{-1} tryptone, 3 g L^{-1} yeast extract, 1 g L^{-1} potassium phosphate dibasic, and 2.5 mL of 40% w/v glucose) supplemented with appropriate antibiotics (chloramphenicol, 5 μg mL^{-1}; neomycin, 5 μg mL^{-1}; tetracycline, 0.5 μg mL^{-1}). *Escherichia coli* (Epi300, Lucigen) was grown at 37°C in Luria Broth (LB) supplemented with chloramphenicol, 15 μg mL^{-1}. *Escherichia coli* ECGE101 (Δ*dapA*) (31) was grown at 37°C in LB media supplemented with DAP, 60 μg mL^{-1}, and appropriate antibiotics (chloramphenicol, 15 μg mL^{-1}, and/or gentamicin, 40 μg mL^{-1}). *Saccharomyces cerevisiae* VL6-48 (ATCC MYA-3666: MATα *his3*-Δ200 *trp1*-Δ1 *ura3*–52 *lys2ade2*–1 *met14 cir*^{0}) was grown at 30°C in 2X YPAD rich medium (20 g L^{-1} yeast extract, 40 g L^{-1} peptone, 40 g L^{-1} glucose, and 80 mg L^{-1} adenine hemisulfate), or in complete minimal medium lacking histidine supplemented with 60 mg L^{-1} adenine sulfate (Teknova Inc.) with 1 M sorbitol.

3.3.2 Plasmid design and construction

All plasmids in this study (Supplemental Table C-1) were constructed from PCR amplified DNA fragments assembled using a yeast spheroplast transformation method as previously described (42). The primers used to amplify the fragments for plasmid assembly (Supplemental Table C-2) contained 20 bp binding and 40 bp of overlapping homology to the adjacent DNA fragment. Following assembly, DNA was isolated from *S. cerevisiae* and the plasmid pool was electroporated into *E. coli* Epi300. Plasmids from individual colonies were screened for correct assembly using multiplex PCR and diagnostic restriction digest, and one final clone was confirmed by next-generation

sequencing (MGH CCIB DNA Core, Massachusetts, USA). All plasmids (pDEINO1-10) have a pCC1BAC-yeast backbone allowing replication and selection in *E. coli* (chloramphenicol) and *S. cerevisiae* (-HIS) with a low-copy *E. coli* origin that can be induced to high copy with arabinose. They also have an origin of transfer (*oriT*) necessary for conjugation. Additional components on each plasmid are specified below. pDEINO1: replicating plasmid constructed by restriction digestion of pAGE3.0 (31) with PacI to insert a synthesized, codon-optimized *cat* gene under the control of a constitutive promoter (drKatA) and an origin of replication for *D. radiodurans* from pRadDEST-GFP and pRAD1 (30), respectively. This plasmid also contains elements for replication and selection in other host organisms, *S. meliloti* and *Phaeodactylum tricornutum,* including an *nptII* marker for *S. meliloti* that is functional in *D. radiodurans*. pDEINO2: nonreplicating plasmid that contains a 1 kb region of homology to the *McrC* gene on the MP1 megaplasmid, amplified from wild-type *D. radiodurans* genomic DNA and a codon-optimized *cat* gene for selection in *D. radiodurans*. pDEINO3-5: replicating plasmids that contain an origin of replication for *D. radiodurans*, a codon-optimized *cat* gene, coupled with selective marker genes *tetR/A* from pAGE2.0 (31), *aadA1* from pAGE1.0 (31), and *aacC1* from pTA-Mob (32), respectively. pDEINO6: built with the same components as pDEINO3, but lacks the codon-optimized *cat* gene. pDEINO7-10: nonreplicating plasmids that contain two 1 kb regions of homology flanking ORF14075, ORF15360, *Mrr*, and ORF2230, respectively, amplified from wild-type *D. radiodurans* genomic DNA. Between the homology regions on the plasmid is a selective marker (*tetR/A* for pDEINO8 and pDEINO9 or *nptII* for pDEINO7 and pDEINO10).

3.3.3 $CaCl_2$ transformation of *D. radiodurans*

For competent cells: A 50 mL culture of *D. radiodurans* was grown to an OD_{600} of 0.2. The culture was transferred to a 50 mL falcon tube and centrifuged at 3000 *g* for 15 min at 4°C. The pellet was resuspended in 10 mL of ice-cold 0.1 M $CaCl_2$ using gentle agitation. The cells were incubated on ice for 30 minutes then centrifuged at 3000 *g* for 15 min at 4°C. The pellet was resuspended in 250 µL of ice-cold 0.1 M $CaCl_2$ 15% glycerol solution using gentle agitation. The competent cells were aliquoted, frozen in a -

80°C ethanol bath and stored at -80°C. *For transformation*: Competent cells were thawed on ice for 15 min. Then, 5 µL of transforming DNA was mixed with 50 µL of competent cells (total DNA concentration was 69 ng of pDEINO10 linear cassette, 1.12 µg of pRAD1, and 460 ng of pDEINO1). The mixture was incubated on ice for 30 min then heat shocked in a 42°C water bath for 45 s. The tubes were returned to ice for 1 min and 1 mL of 2X TGY media was added to each tube. The recovery cultures were transferred to a 50 mL falcon tube and grown with shaking at 30°C for 2 hours at 225 rpm. Finally, 300 µL of the transformation mixture was plated on TGY media supplemented with chloramphenicol 3 µg mL^{-1} or neomycin 5 µg mL^{-1} and incubated at 30°C for 2-3 days. Colonies were counted manually.

3.3.4 Conjugation from *E. coli* to *D. radiodurans*

The *E. coli* ECGE101 Δ*dapA* donor strain (31), harboring pTA-Mob (32) and pDEINO1, and a control strain, harboring only pDEINO1, were grown at 37°C overnight in 5 mL of LB media supplemented with DAP 60 µg mL^{-1}, gentamicin 40 µg mL^{-1} (donor only), and chloramphenicol 15 μg^{-1}. The saturated *E. coli* cultures were diluted 1:50 into 50 mL of the same media and grown for ∼2 h to an OD_{600} of 0.6. The *D. radiodurans* R1 recipient strain was grown at 30°C overnight in TGY media to an OD_{600} of 0.6. Donor, control, and recipient cultures were transferred to 50 mL falcon tubes and centrifuged at 3000*g* for 20 min at 4°C. The supernatant was discarded, and cell pellets were resuspended in 300 µL of TGY media. Then, 50 µL of *E. coli* donor or control cultures and 50 µL of *D. radiodurans* recipient culture were directly mixed and spread on a conjugation plate (TGY media supplemented with DAP 60 µg mL^{-1}), previously dried for 1 h. Conjugation plates were incubated at 30°C for 3 h, then cells were scraped off with 1 mL of TGY media and adjusted to a final volume of 1 mL in a microfuge tube. A 10-fold serial dilution was performed in TGY media, and 100 µL was plated on both selective (TGY supplemented with chloramphenicol 5 µg mL^{-1}) and nonselective (TGY) plates. Nonselective and selective plates were incubated at 30°C for 2 days and 3 days, respectively, and colonies were counted manually.

3.3.5 Additional conjugation controls

Conjugation to *D. radiodurans* was performed as described in the methods section with *E. coli* cells harbouring pTA-Mob and pDEINO1, with *E. coli* cells harbouring pTA-Mob2.0 Δ*traI* and pDEINO1, with 1 µg of pDEINO1 plasmid DNA directly mixed with recipient cells, or with recipient cells only. All conjugation conditions were performed with and without the addition of DNase by adding 2 units of TurboDNase to the cell mixture prior to plating on conjugation plates and again before plating on selective plates. The selective plates (TGY Cm 5 µg mL^{-1}) were incubated for three days at 30°C. Colonies were counted manually.

3.3.6 Transconjugant plasmid isolation

D. radiodurans transconjugant colonies (10 from each experiment, 30 total) were passed twice on TGY plates supplemented with chloramphenicol (5 µg mL^{-1}), then inoculated into 5 mL of the same media and grown overnight at 30°C. Alkaline lysis was performed using 3 mL of saturated culture as previously described (42), and the DNA was electroporated into *E. coli* Epi300 cells. The plasmids were induced to high copy number in *E. coli* in 5 mL of LB media supplemented with chloramphenicol (15 µg mL^{-1}) and arabinose (100 µg mL^{-1}) for 8 hours before isolating for analysis using the BioBasic EZ10 Spin Column Miniprep Kit.

3.3.7 Transconjugant plasmid analysis

Plasmids were analyzed by multiplex PCR with primers listed in Supplemental Table C-2 using 10 µL of Qiagen MPX, 3 µL of primer mix, 6 µL of water and 1 µL of template DNA diluted to 1 ng $µL^{-1}$. Thermocycler conditions were as follows: 95°C 5 min, 35 cycles of: 94°C 30 s, 60°C 90 s, and 72°C 10 s, then 72°C 10 min. Gel electrophoresis was performed using 2 µL of the PCR reaction on a 2% agarose gel at 90 kV for 60 min

which was stained with ethidium bromide for visualization. Transconjugant plasmids were further analyzed with a diagnostic restriction digest using 0.2 µL of EcoRI-HF, 5 µL of ~100 ng μL^{-1} plasmid DNA, 2 µL Cutsmart buffer and 12.8 µL of water incubated at 37°C for 2 h. Gel electrophoresis was performed using 10 µL of the digest on a 1% agarose gel at 100 kV for 120 min. Two transconjugant plasmids were analyzed by next-generation sequencing (MGH CCIB DNA Core, Massachusetts, USA) and compared to the original pDEINO1 sequence using the Benchling alignment tool (Benchling [Biology Software] (2021). Retrieved from https://benchling.com).

3.3.8 Spot plating *D. radiodurans*

D. radiodurans was grown overnight in 5 mL cultures of TGY media supplemented with the appropriate antibiotics for plasmid maintenance. The cultures were diluted to an OD_{600} of 0.1 before performing 10-fold serial dilutions in TGY media up to 10^{-5} dilution. Then, 5 µL of each dilution was plated on TGY plates supplemented with appropriate antibiotics and incubated at 30°C for 2-3 days.

3.3.9 Plasmid stability assay of *D. radiodurans* transconjugants

One *D. radiodurans* transconjugant harbouring pDEINO1 was plated on TGY supplemented with chloramphenicol (5 µg mL^{-1}) to obtain single colonies. A single colony was inoculated in 50 mL of the same media and grown with shaking to an OD_{600} of 0.5 at 30°C. The culture was diluted to an OD_{600} of 0.1 and 100 µL of a dilution series of 10^{-1} to 10^{-5} was plated on nonselective (TGY) and selective (TGY supplemented with chloramphenicol 5 µg mL^{-1}) media. These plates were incubated at 30°C for 3 days and colonies were counted manually. From the diluted culture (OD_{600} of 0.1), 50 µL was used to subculture into 50 mL of fresh nonselective media and grown with shaking at 30°C for an additional ~13 generations to an OD_{600} of 0.5. This process was repeated for a total of ~40 generations. The ratio of *D. radiodurans* colonies on selective plates to colonies on non-selective plates after each subculturing event was reported as an indicator of plasmid

loss. Additionally, 100 colonies from the selective and nonselective plates were struck onto TGY plates supplemented with chloramphenicol each day and the number of colonies unable to grow on selection was used as a second strategy to determine plasmid loss.

3.3.10 Cloning the *D. radiodurans* MP1 megaplasmid

Conjugation from *E. coli* to *D. radiodurans* was performed as described above using *E. coli* ECGE101 pTA-Mob pDEINO2 as the donor and *D. radiodurans* as the recipient, with *D. radiodurans* grown to an OD_{600} of 2 rather than 0.6. *D. radiodurans* transconjugants were selected by plating 100 µL onto 10 TGY plates supplemented with chloramphenicol (5 µg mL^{-1}). Three transconjugants were isolated as described above in Section 3.3.6 and analyzed by multiplex PCR using pDEINO2 multiplex primers as described below in Section 3.3.11. The DNA was transformed into *E. coli* Epi300 and, following induction in *E. coli*, plasmids were isolated using alkaline lysis. The cloned MP1 plasmids were analyzed by two multiplex PCRs, one amplifying fragments within the conjugative nonreplicating plasmid (pDEINO2), and one amplifying fragments within the MP1 megaplasmid. Primers used for the pDEINO2 and MP1 megaplasmid multiplex PCR are listed in Supplemental Table C-2. The plasmids were further analyzed by two diagnostic restriction digests using MfeI-HF and NheI-HF. Finally, total DNA from one clone was isolated using the Monarch Kit for HMW DNA Extraction from Bacteria (NEB#T3060) and confirmed by Nanopore sequencing as described below in Section 3.3.13.

3.3.11 Gene deletions

Conjugation from *E. coli* to *D. radiodurans* was performed as described above using *E. coli* ECGE101 pTA-Mob harboring pDEINO7 (ORF14075), pDEINO8 (ORF15360), pDEINO9 (*Mrr*), or pDEINO10 (ORF2230) as the donor and *D. radiodurans* as the recipient. *D. radiodurans* transconjugants were selected on TGY supplemented with

neomycin (5 μg mL^{-1}) for pDEINO7 and pDEINO10 or tetracycline (0.5 μg mL^{-1}) for pDEINO8 and pDEINO9. Transconjugant DNA was isolated as described above in Section 3.3.6 and analyzed by multiplex PCR with primers listed in Supplemental Table C-2 using 10 μL of Qiagen MPX, 2 μL of primer mix, 6 μL of water, 1 μL of DMSO, and 1 μL of alkaline lysis DNA. Thermocycler conditions were as follows: 95°C 5 min, 30 cycles of 94°C 30 s, 60°C 90 s, and 72°C 90 s, then 72°C 10 min. Gel electrophoresis was performed using 2 μL of the PCR reaction on a 2% agarose gel at 110 kV for 50 min.

3.3.12 *D. radiodurans* R1 genomic DNA isolation

D. radiodurans genomic DNA was isolated in agarose plugs using the Bio-Rad CHEF Genomic DNA Plug Kit (Bio-Rad, CAT#170-3592) as previously described (42) with an adapted protocol. To prepare the plugs, 50 mL of *D. radiodurans* culture was grown to OD_{600} of 1.0, chloramphenicol (100 μg mL^{-1}) was added and the culture was grown for an additional hour. The culture was centrifuged at 5000*g* for 5 min at 4°C. Cells were washed once with 1 M sorbitol in 1.5 mL Eppendorf tubes and centrifuged at 4000 RPM for 3 min at room temperature. The supernatant was removed, and cells were resuspended in 600 μL of protoplasting solution (for 10 mL: 4.56 mL of SPEM solution, 1000 μl Zymolyase-20T solution (50 mg mL^{-1} dissolved in H_2O), 400 μL lysozyme (25 mg mL^{-1}), 400 μl hemicellulase (25 mg mL^{-1}), 50 μl β-mercaptoethanol). The cell suspension was incubated for 5 min at 37°C and mixed with an equal volume of 2% low-melting-point agarose in 1×TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA) which was equilibrated at 50°C. Aliquots of 95 μL were transferred into plug molds (Bio-Rad, CAT#170–3713) and allowed to solidify for 10 min at 4°C. Next, plugs were removed from the molds into a 50 mL conical tube containing 5 mL of protoplasting solution and incubated for 45 min at 37°C. Plugs were washed with 25 mL of wash buffer (20 mM Tris, 50 mM EDTA, pH 8.0), and then incubated in 5 mL Proteinase K buffer (100 mM EDTA (pH 8.0), 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine, 1 mg ml^{-1} Proteinase K) for 24 h at 50°C. The plugs were washed four times with 25 mL of wash buffer for 30 min each at room temperature and incubated in wash buffer overnight at 4°C. The next day, the plugs were washed four times with 10X-diluted wash buffer for 30

min each, then stored at 4°C in 10X-diluted wash buffer. To isolate DNA from the plugs, two plugs were transferred to a 1.5 mL Eppendorf tube and were washed once with 10X-diluted wash buffer for 1 h, and once with TE buffer for 1 h. The TE buffer was removed and the tube was incubated at 42°C for 10 min, followed by 65° for 10 min. The tube was returned to 42° for 10 min then 50 µL of TE buffer and 3 µL of β-agarase was added and flicked gently to mix. The plugs were incubated at 42°C for 1 h. Another 50 µL of TE buffer was added to the tube and incubated for 2 h at 42°C. The DNA purity was confirmed by gel electrophoresis of 1 µL on a 1% agarose gel.

3.3.13 DNA sequencing and analysis

D. radiodurans R1 (ENA: PRJEB48130). Genomic DNA isolation was performed using agarose plugs as described above. The library was prepared using the SQK-LSK109 kit according to the manufacturer's protocol. An R9.4.1 flow cell was used for sequencing. Basecalling was performed using Guppy v4.2.2 (Oxford Nanopore Technologies) in high-accuracy mode. Genome assembly was performed with Flye v2.8.1 (44). The assembly was polished with one round of Racon (45) and one round of Medaka (Oxford Nanopore Technologies). *D. radiodurans R1 Δ14075 Δ15360.* Genomic DNA isolation was performed using agarose plugs as described above. The DNA was sequenced using an RBK004 kit on an Oxford Nanopore MinIon R9.4.1 flow cell according to the manufacturer's protocol. Reads were basecalled using Guppy v5.015 in high-accuracy mode. *E. coli pDEINO2-MP1*. The library was prepared using the SQK-LSK109 kit and the EXP-NBD104 native barcoding kit according to the manufacturer's protocol. An R9.4.1 flow cell was used for sequencing. Basecalling was performed using Guppy v5.0.7 (Oxford Nanopore Technologies) in high-accuracy mode. Genome assembly was performed with Flye v2.8.1 in –meta mode (44). The assembly was polished with one round of Racon (45) and one round of Medaka (Oxford Nanopore Technologies). Plasmids were extracted from the final assemblies, and aligned using minimap2 (46) against the expected sequences to identify any mutations.

3.4 References

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Chapter 4

4 SLICER: Seamless Loss of Integrated Cassettes Using Endonuclease Cleavage and Recombination in *Deinococcus radiodurans*

4.1 Introduction

Deinococcus radiodurans is emerging as a bacterial platform for synthetic biology and industrial applications including bioremediation and antioxidant biosynthesis (1–3). *D. radiodurans* is a polyextremophile with exceptional resistance to the lethal effects of ionizing and ultraviolet (UV) radiation, desiccation and other DNA-damaging agents (4,5). This resistance has been linked to the superior homologous recombination and DNA repair mechanisms of this bacterium (6), which have been shown to repair the genome as well as exogenous plasmid DNA following irradiation (7). By exploiting this machinery, *D. radiodurans* has the potential to be a synthetic biology chassis with superior DNA assembly capabilities. Currently, large DNA constructs and whole genomes are most commonly assembled in *Saccharomyces cerevisiae* (8,9). *D. radiodurans* could complement this method as a GC-rich DNA assembly platform.

To achieve DNA assembly, a highly efficient transformation method for *D. radiodurans* is required. We recently demonstrated conjugation as an efficient DNA delivery method; however, for the development of an assembly system, a method for simultaneous transformation of multiple linear fragments will be necessary (10). Although protocols for chemical transformation of *D. radiodurans* exist, the current strains and protocols do not allow for efficient transformation, as demonstrated by the inefficient transformation of small (~6 kb) or large (*i.e.,* >20 kb) plasmids (10). We hypothesized that the presence of active restriction-modification (R-M) systems could be one possible reason for the low transformation efficiencies obtained (11).

Many microorganisms possess R-M systems as part of the bacterial immune system, protecting against foreign DNA molecules (12). Putative R-M systems in *D.*

radiodurans R1 have been identified throughout its two chromosomes and two plasmids, which has been summarized on REBase (http://rebase.neb.com/); a database of information on restriction enzymes and methyltransferases involved in R-M systems (13). The genome contains four Type II and two Type IV R-M systems containing restriction endonucleases, as well as a lone methyltransferase on the CP1 plasmid. Some of the R-M systems have been further characterized empirically (14–16).

Further evidence that R-M systems are preventing efficient transformation was reported by Meima and Listrom (2001) following the transformation of *D. radiodurans* with plasmids isolated from methylation-proficient and -deficient strains of *Escherichia coli.* Researchers found that unmethylated plasmids resulted in 50-750-fold improvements in transformation efficiency, compared to *E. coli* methylated plasmids (17). The authors concluded that methylation-specific nucleases are likely restricting the transformed DNA, and that inactivation of the R-M systems in *D. radiodurans* could result in an improved bacterial chassis for genetic engineering (17). Therefore, to improve transformation of *D. radiodurans*, we sought to create a *D. radiodurans* strain lacking R-M systems by creating seamless R-M gene knockouts. To achieve this, we developed the Seamless Loss of Integrated Cassettes using Endonuclease Cleavage and Recombination (SLICER) method in *D. radiodurans*, and demonstrated its use by sequential deletion of four of the six R-M systems. Following transformation of a neomycin selectable marker to replace the fifth R-M system, a final *D. radiodurans* strain lacking five of the six R-M systems was created. Deletion of these systems did not result in growth inhibition of these *D. radiodurans* strains. This partially restriction minus strain also resulted in slightly increased transformation efficiency of a small (~6 kb) plasmid.

4.2 Results and Discussion

4.2.1 Design of the *D. radiodurans* seamless deletion strategy

We sought to create a seamless gene deletion strategy to knock out a gene of interest (GOI) in the *D. radiodurans* genome. In order to exploit *D. radiodurans'* high propensity for homologous recombination (HR), we based our strategy on a method developed for

another HR-proficient microorganism, *S. cerevisiae*. The tandem repeat coupled with endonuclease cleavage (TREC) method developed by Noskov et al. (2010), is a seamless modification method demonstrated to engineer a *Mycoplasma genitalium* genome hosted in yeast (18). We developed a modified TREC method for *D. radiodurans* to create seamless deletions, entitled SLICER. The complete SLICER method is depicted in Figure 4-1.

A nonreplicating multi-host shuttle plasmid, termed the Seamless Deletion plasmid (pSD), is first built specifically for the targeted DNA region or GOI (Figure 4-2A). The main component of this plasmid will herein be referred to as the Seamless Deletion (SD) cassette, which contains a neomycin resistance gene and *lac*Z (β-galactosidase) marker for selection and screening in *D. radiodurans* flanked by two regions homologous to the *D. radiodurans* genome. These 1 kb homology regions are the sequences upstream and downstream of the target sequence. Following the first homology region, there is an 18-bp I-*Sce*I endonuclease recognition site and prior to the second homology region there is a duplication of the last 80 bp of homology region 1. A second selective marker for *D. radiodurans* (either streptomycin or tetracycline resistance gene) is located on the plasmid backbone outside of the SD cassette.

The first step in the SLICER method is the integration of the SD cassette into the *D. radiodurans* genome at the target locus. Polymerase Chain Reaction (PCR) amplification of the SD cassette results in a linear DNA fragment approximately 6750 bp in size. Following transformation of the SD cassette into *D. radiodurans*, homologous recombination of the two homology regions with the corresponding genomic regions results in integration of the SD cassette into the *D. radiodurans* genome, replacing the target gene. *D. radiodurans* transformants containing the cassette can be selected on TGY media supplemented with neomycin (5 μg mL^{-1}) and X-Gal (40 μg mL^{-1}) and appear blue in colour due to the expression of the *lacZ* gene. The resulting strain is referred to as *D. radiodurans* + SD.

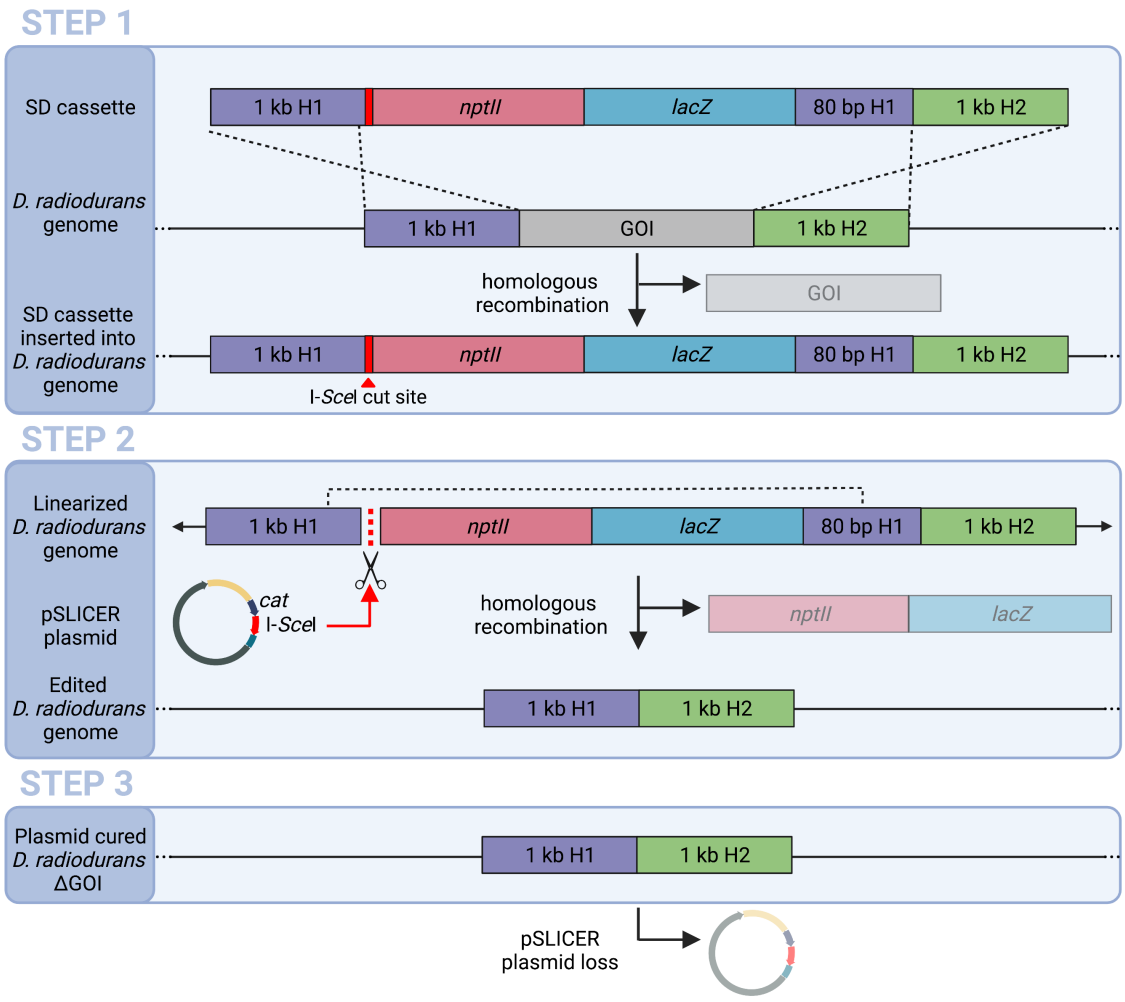


Figure 4-1. Overview of the SLICER method. STEP 1: The SD cassette contains *nptII* and *lacZ* genes for selection and visual screening following chemical transformation of the SD cassette into *D. radiodurans*. Homologous recombination of the 1 kb H1 and H2 regions occurs with the *D. radiodurans* genome resulting in integration of the SD cassette into the genome replacing the target gene of interest (GOI). **STEP 2:** The pSLICER plasmid is conjugated into *D. radiodurans* where it expresses an I-*Sce*I homing endonuclease that cuts at the 18-bp I-*Sce*I restriction site within the SD cassette. This double-strand break triggers homologous recombination between the duplicated 3' 80 bp of H1, removing the *nptII* and *lacZ* markers. **STEP 3:** Finally, plasmid curing to remove the pSLICER plasmid results in a marker-free *D. radiodurans* ΔGOI strain. SD, seamless deletion; H1, homology region 1; H2, homology region 2; GOI, gene of interest; *nptII*, neomycin resistance gene; *lacZ*, β-galactosidase. Created with BioRender.com.

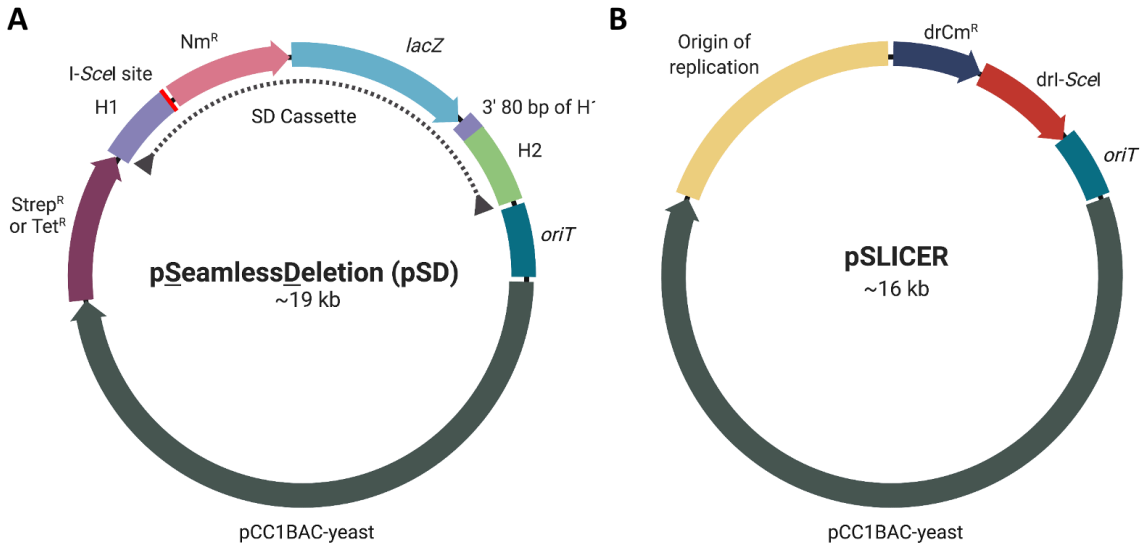


Figure 4-2. Plasmid maps of pSD and pSLICER. (A) Schematic of a representative seamless deletion plasmid (pSD) with the general components contained on pSD1, pSD2, pSD3 and pSD4: H1, homology region 1; I-*Sce*I site, I-*Sce*I endonuclease recognition site; Nm^R, neomycin resistance gene (*nptII)*; *lacZ*, β-galactosidase gene; 3' 80 bp of homology 1, duplication of the last 80 bp of H1; H2, homology region 2; *oriT*, origin of transfer; pCC1BAC-yeast, backbone for selection and replication in yeast and *E. coli;* $Strep^R$, streptomycin resistance gene (*aadA1*); Tet^R, tetracycline resistance gene (*tetR/A*). The SD cassette is indicated with a dotted line. (B) Schematic of a pSLICER: Origin of replication, origin of replication for *D. radiodurans;* $drCm^R$, chloramphenicol resistance gene codon-optimized for *D. radiodurans*; drI-*Sce*I, I-*Sce*I endonuclease codon-optimized for *D. radiodurans*. Created with BioRender.com.

Rather than using a screening marker like *lacZ*, the TREC method employs counterselection by incorporating 5-fluoroorotic acid (5-FOA) on the integrating cassette. *SacB* is another counterselectable marker that originates from *Bacillus subtilis* and encodes levansucrase. In the presence of sucrose, levansucrase catalyzes the hydrolysis of sucrose to fructose followed by levan sucrose which is harmless in most Gram-positive bacteria but can be lethal when expressed in Gram-negative bacteria (19). This promotes the elimination of the *sacB* gene from bacterial strains, and ultimately the removal of the integrated cassette. Use of the *sacB* counterselectable marker in *D. radiodurans* was previously reported by Pasternak et al. (2010), however use of this marker in the SD cassette was unsuccessful in our hands (data not shown) (20). Similar difficulties with this counterselectable marker were reported in *Deinococcus geothermalis* (21).

The second step in the SLICER method is the removal of the SD cassette facilitated by the introduction of the pSLICER plasmid. We constructed the replicating helper plasmid, pSLICER, containing a codon-optimized I-*Sce*I endonuclease, an origin of replication and a chloramphenicol selective marker for *D. radiodurans* (Figure 4-2B). I-*Sce*I is an intron-encoded homing endonuclease originating from the mitochondria of *S. cerevisiae* (22). We chose this enzyme because there are no recognition sites present in the wild-type genome of *D. radiodurans*. The I-*Sce*I endonuclease was designed under the regulation of the PDR_2508 promoter and terminator set identified by Chen et al. (2019) as it was shown to have high expression in *D. radiodurans* but low expression in *E. coli* (23). This plasmid is then transformed into an *E. coli* Δ*dap*A strain harbouring the conjugative plasmid pTA-Mob. Conjugation of the pSLICER plasmid from the *E. coli* donor strain to *D. radiodurans* + SD is then performed. Expression of the I-*Sce*I endonuclease leads to cleavage at the I-*Sce*I recognition sequence within the SD cassette which stimulates homologous recombination between homology region 1 and the 80-bp duplicated region. Transconjugants can be selected on TGY media supplemented with chloramphenicol (3 μg mL^{-1}) and X-Gal. Contrary to the previous screening, transconjugants that have had the SD cassette removed via homologous recombination should appear pink since they have lost the *lac*Z gene. The resulting strain is referred to as *D. radiodurans* + SLICER.

The final step in the SLICER method is to cure the strain of the pSLICER plasmid. The *D. radiodurans* + SLICER strain is grown in nonselective media overnight and dilutions are subsequently spot plated on nonselective media. Resulting single colonies are then struck on nonselective media as well as TGY media supplemented with either chloramphenicol or neomycin. The colonies are confirmed to be cured of the plasmid when growth is observed on nonselective plates but not on selective plates. At the end of the seamless deletion process, the resulting *D. radiodurans* ΔGOI strain will have the target gene deleted with no remnants of the process remaining in the genome or the cell. The entire SLICER method can be completed in approximately 2 weeks and the step-by-step protocol is summarized in Figure 4-3.

4.2.2 Seamless deletion of four *D. radiodurans* R-M system genes

Using the seamless deletion strategy outlined above, we performed the sequential deletion of four out of the six R-M system genes in the *D. radiodurans* genome (Figure 4-4), with the fifth R-M system (ORF2230) subsequently deleted using homologous-recombination based integration of a neomycin marker. Four nonreplicating SD plasmids, named pSD1-pSD4, were built for each R-M target gene: ORF14075, *Mrr*, ORF15360, and *Mrr2*, which will herein be called RM1, RM2, RM3 and RM4, respectively (Figure 4-2). These target genes were named numerically in the order that they were used to generate deletions. Each plasmid contains the same elements apart from the homology regions, which are specific to each target gene. To simplify the SD protocol, we modified several steps in the preparation of $CaCl_2$-competent cells for the heat shock transformation protocol previously described (10). All simplifications of the protocol produced a similar number of *D. radiodurans* transformants upon transformation with a linear DNA cassette. Therefore, all subsequent transformations of the SD cassette were performed with the simplest version of the protocol (Figure 4-5).

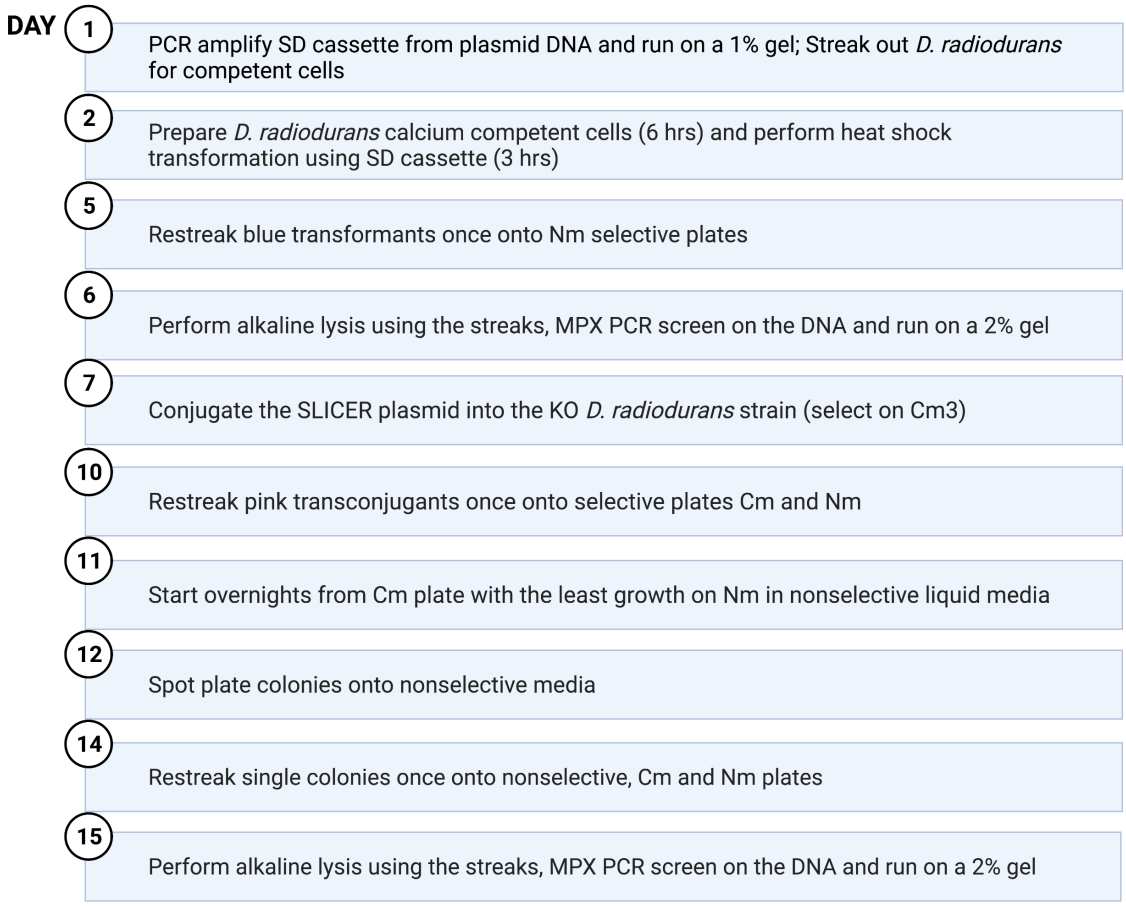


Figure 4-3. Step-by-step SLICER protocol. Laboratory protocol for the SLICER method, which can be used to create a seamless gene deletion in *D. radiodurans* in approximately 2 weeks. Created with BioRender.com.

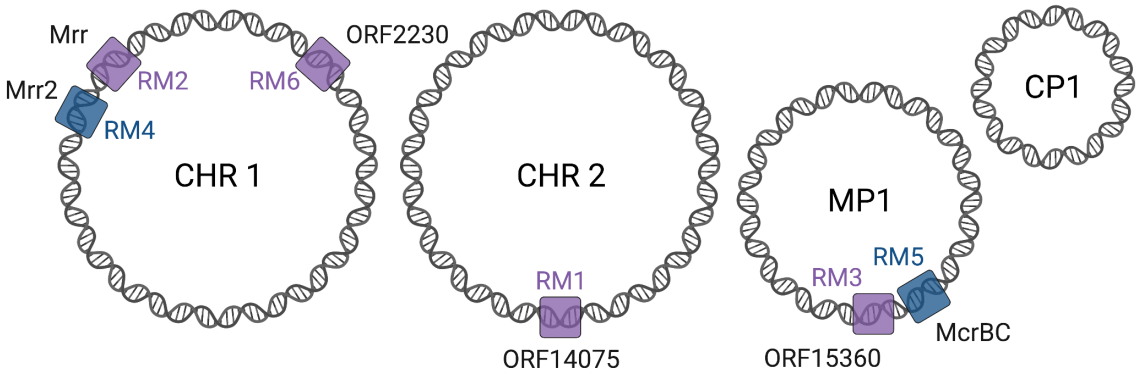


Figure 4-4. *D. radiodurans* restriction-modification systems. Identified R-M systems and relative positions in the *D. radiodurans* genome (*ie.,* chromosome 1 (CHR 1), chromosome 2 (CHR 2), MP1 megaplasmid, and CP1 plasmid). Type II R-M systems are depicted in purple and Type IV R-M systems are depicted in blue as summarized by Li et al. (2019) (15). RM1-6 indicates the order in which these genes were targeted for deletion. Created with BioRender.com.

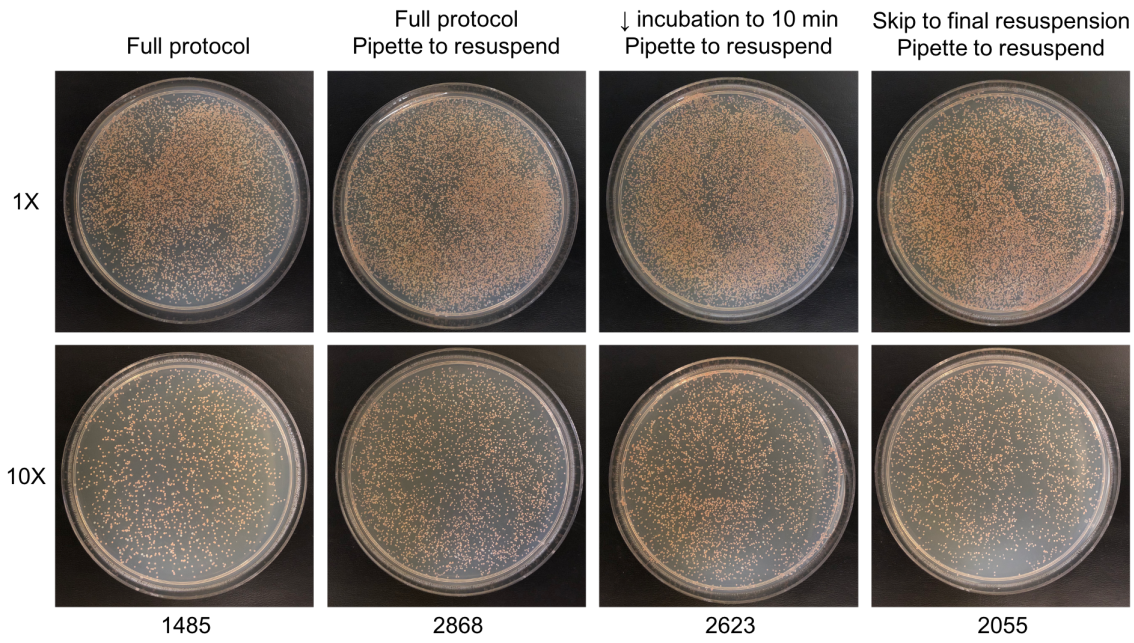


Figure 4-5. Testing simplified $CaCl_2$ heat shock transformation for *D. radiodurans*. Transformation of *D. radiodurans* with a linear ~6.7 kb cassette using $CaCl_2$-competent cells prepared four ways. From left to right the transformation protocol is simplified starting with the full protocol and ending with a protocol where all resuspension and incubation steps are removed apart from the final resuspension. Transformed cultures were plated on TGY media supplemented with neomycin (5 μg mL^{-1}). Undiluted and 10-fold diluted plates are shown with transformant colony counts for the 10-fold plate provided at the bottom.

Gene deletion analysis of all four knockout strains is shown in Figure 4-6 for the deletion of RM1, RM2, RM3 and RM4 resulting in the creation of *D. radiodurans* ΔRM1, ΔRM1-2, ΔRM1-3 and ΔRM1-4 strains, respectively. Following Step 1, 2 and 3 of the SLICER protocol (as outlined in Figure 4-1), the *D. radiodurans* genome was analyzed to confirm insertion of the SD cassette, removal of the SD cassette, and curing of the pSLICER plasmid. Analysis was conducted by spot plating dilutions on nonselective media, media supplemented with neomycin and media supplemented with chloramphenicol, all of which contained X-Gal (Figure 4-6B). In addition, multiplex PCR analysis was performed on DNA extracted from one individual colony for each seamless deletion event (RM1-4) (Figure 4-6C). If present in the examined DNA, the multiplex PCR should amplify a 150 bp amplicon at a non-target site in the *D. radiodurans* genome, a 300 bp amplicon within the neomycin marker on the SD cassette, a 500 bp amplicon within the target gene (RM1-4), and/or a 650 bp amplicon within the pSLICER backbone. The position and size of the expected amplicons following each step of the seamless deletion strategy are depicted in Figure 4-6A. For the RM1 multiplex, a wild-type genomic DNA control is used, and for all subsequent multiplex analyses the cured strain from the previous deletion was used as a control (*e.g.*, *D. radiodurans* ΔRM1 DNA is used as a control for analysis of *D. radiodurans* ΔRM1-2).

From the analyses of the RM1-RM4 deletions (Figure 4-6B,C), we observed that the wild-type *D. radiodurans* strain was only able to grow on nonselective media, appeared pink in colour, and the PCR results showed amplification of the genomic DNA control and target gene amplicons. Multiplex PCR performed on the pSD1-4 plasmids containing the SD cassette showed amplification of the neomycin marker and backbone amplicons. Following integration of the SD cassette, *D. radiodurans* + SD was able to grow on the nonselective and neomycin supplemented media, and appears blue in colour on both. The PCR results show amplification of the genomic control and notably, there is no amplification of the target gene amplicon. After conjugating in the pSLICER plasmid, *D. radiodurans* + SLICER is able to grow on nonselective and chloramphenicol supplemented media, but not neomycin supplemented media. With the loss of the SD

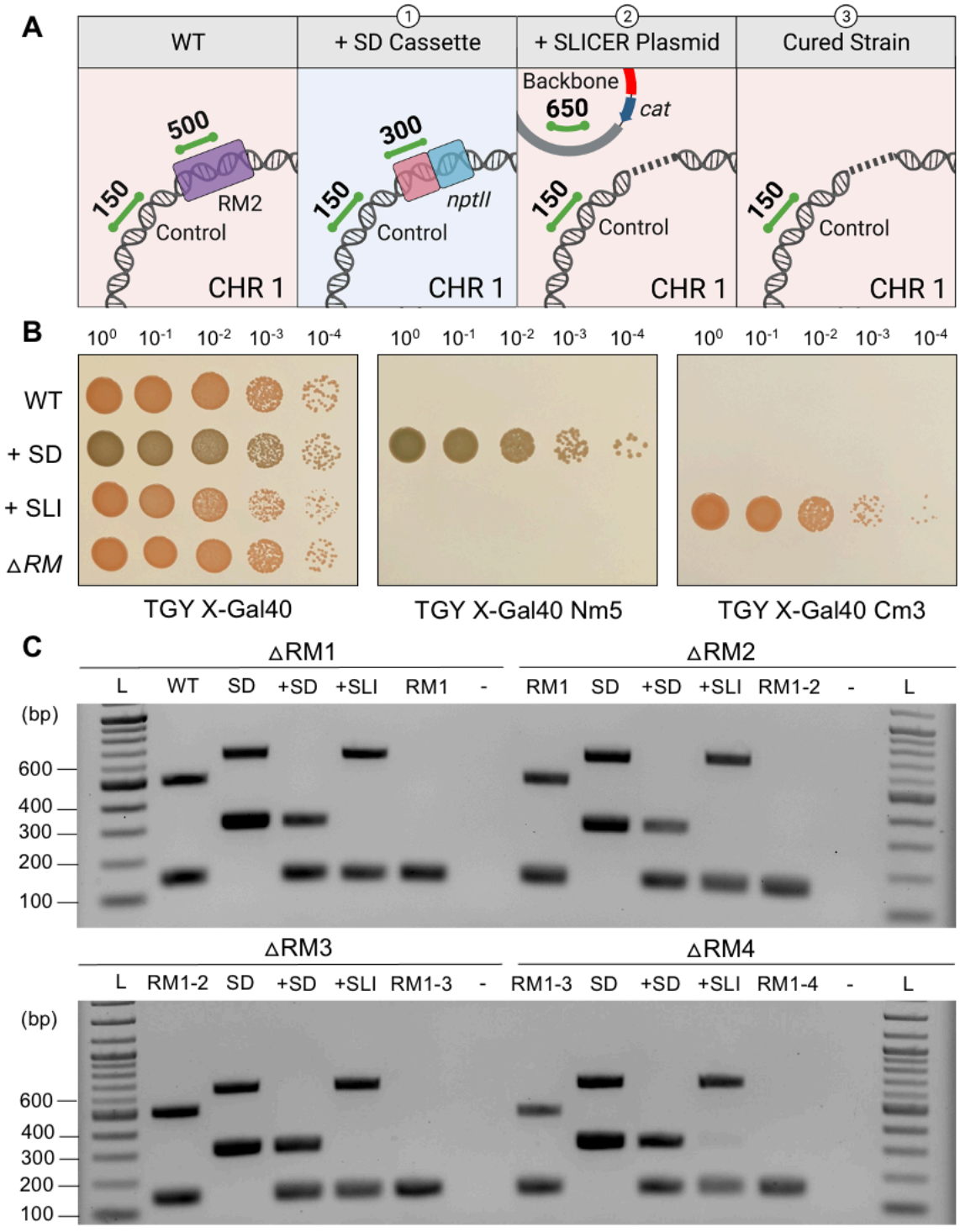


Figure 4-6. Seamless deletion of RM1-4 genes in *D. radiodurans*. (A) Schematic of the multiplex PCR amplicons present in *D. radiodurans* strains: wild type, with the SD cassette integrated at the RM2 locus, with the addition of the SLICER plasmid seamlessly removing the SD cassette, and the final *D. radiodurans* ΔRM2 strain. Amplicons are shown as green lines with the corresponding size in bp written on top. Created

with BioRender.com. (B) Spot plates of 10-fold serial dilutions of the same strains listed in (A). All plates contain X-Gal 40 μg mL^{-1}. (C) Gel electrophoresis of multiplex PCR results from all steps in the creation of the four seamless R-M gene deletions. The PCR for each deletion (ΔRM1, ΔRM2, ΔRM3, ΔRM4*)* was performed on DNA from a single *D. radiodurans* colony in the order depicted in (A): *D. radiodurans* gDNA (WT), following integration of the SD cassette (+SD), following conjugation of the SLICER plasmid (+SLI), and following plasmid curing (RM1, RM1-2, RM1-4, and RM1-4). Controls include the SD plasmid DNA extracted from *E. coli* (SD) and wild-type *D. radiodurans* gDNA (WT). For the RM1 multiplex, a wild-type (WT) genomic DNA control is used, and for all subsequent multiplex analyses the cured strain from the previous deletion was used as a control (RM1, RM1-2, RM1-3). Expected amplicon sizes are approximately 150 bp for the *D. radiodurans* gDNA control, 300 bp for *nptII* in the SD cassette, 500 bp for the R-M gene, and 650 bp for the SLICER plasmid backbone. L, 1 kb plus ladder.

cassette, the colonies once again appear pink. The PCR results show amplification of the genomic control and backbone amplicons. Notably, there is no amplification of the neomycin marker amplicon. Finally, curing of the pSLICER plasmid from *D. radiodurans* ΔRM only allows for growth on the nonselective plate as the strain no longer contains the SD cassette or the pSLICER plasmid, and colonies appear pink as a result. Multiplex PCR shows amplification only of the genomic control amplicon, indicating that the pSLICER plasmid was successfully cured.

Following the fourth deletion, the fifth R-M system was deleted using homologous-recombination based integration of a neomycin marker using the cassette from pDEINO10 previously used to knockout ORF2230 (10). The final *D. radiodurans* ΔRM1-5 Nm^R strain has the majority of the R-M systems removed and can be propagated with neomycin selection (Figure 4-7A). Further confirmation that all four genes (RM1-4) have been seamlessly deleted in the *D. radiodurans* ΔRM1-4 strain was performed using multiplex PCR (Figure 4-7B). Genomic DNA extracted from a single *D. radiodurans* ΔRM1-4 colony was used as a template for the RM1, RM2, RM3, and RM4 multiplexes. These results verify that the *D. radiodurans* ΔRM1-4 strain has all four of the target genes deleted: ORF14075, *Mrr*, ORF15360, and *Mrr2* as none of the amplicons residing in the target gene were amplified from genomic DNA. The sixth R-M system that has not yet been deleted is *mcrBC* on the MP1 megaplasmid. Meima and Lidstrom (2001) created a *D. radiodurans* mutant with an insertion in the *mcrB* gene, which did not lead to an increase in transformation efficiency (17). The low transformation efficiency in *D. radiodurans* may be the result of multiple active R-M systems; therefore, it is unlikely that there would be an improvement by deleting a single system.

4.2.3 Evaluation of I-*Sce*I endonuclease function in SLICER

We then sought to verify that the codon-optimized I-*Sce*I endonuclease encoded on the pSLICER plasmid was not only functional in *D. radiodurans* but is necessary for the success of the SLICER method. To determine the frequency of SD cassette loss from the *D. radiodurans* genome following integration, dilutions of *D. radiodurans* ΔRM1-4

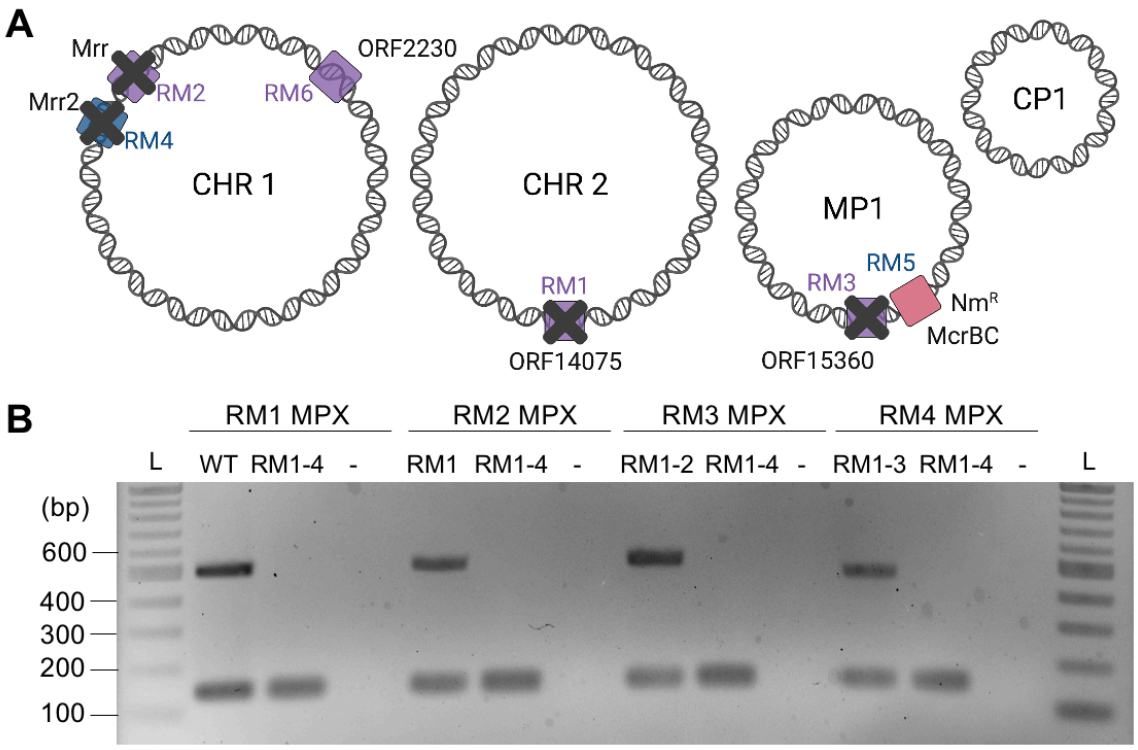


Figure 4-7. *D. radiodurans* ΔRM1-4 strain multiplex PCR analysis. (A) Schematic representation of the *D. radiodurans* ΔRM1-5 Nm^R genome with the first four R-M genes deleted (RM1, RM2, RM3, RM4) as indicated by grey X's, and the fifth R-M system (RM5) replaced with a neomycin marker (Nm^R) as indicated in pink. Created with BioRender.com. (B) Gel electrophoresis of multiplex PCR performed on DNA extracted from a single *D. radiodurans* ΔRM1-4 colony for each of the four R-M gene knockouts (RM1-4) that should be seamlessly deleted in this strain. For the RM1 multiplex, a wild-type (WT) genomic DNA control is used, and for all subsequent multiplex analyses the cured strain from the previous deletion was used as a control (RM1, RM1-2, RM1-3). A negative control (-) where water was used in place of template was also included. Expected amplicon sizes are approximately 150 bp for the *D. radiodurans* gDNA control and 500 bp for the R-M gene, if present. L, 1 kb plus ladder.

Nm^R, harbouring the SD cassette, were plated on nonselective and selective media (Figure 4-8A). Due to the presence of *lacZ* in the SD cassette, blue colonies should be indicative of those carrying the SD cassette while pink colonies indicate loss of the cassette. The percentage of *D. radiodurans* colonies that appeared pink with and without antibiotic selection were 1.1% and 2.1%, respectively, indicating the occurrence of natural SD cassette loss or mutation following propagation. The pink colonies obtained from both nonselective and selective plates were further analyzed by streaking them onto selective media (data not shown). All colonies were able to grow on selective media, indicating that while these colonies appeared to have lost or mutated the *lacZ* gene, the neomycin marker in the SD cassette was still functional. As such, the integrated SD cassettes appear to be quite stable and spontaneous loss of these cassettes could not be easily obtained by plating cultures without selective pressure.

As further confirmation that the I-*Sce*I endonuclease is required for excision of the SD cassette in the SLICER method, conjugation of pSLICER and a control plasmid, pDEINO1, was performed to *D. radiodurans* ΔRM1-4 Nm^R with the integrated SD cassette (Figure 4-8B). The pDEINO1 plasmid contains all of the same components as pSLICER including a *D. radiodurans* origin of replication and chloramphenicol marker, but lacks the I-*Sce*I endonuclease (10). When this plasmid was conjugated to *D. radiodurans*, all transconjugant colonies appeared blue, indicating that they still harbored the SD cassette. Conversely, when the pSLICER plasmid was conjugated to *D. radiodurans*, all transconjugant colonies appeared pink, indicating that the SD cassette had been lost. This allowed us to conclude that the codon-optimized I-*Sce*I endonuclease is functional in *D. radiodurans* and is essential for SD cassette excision.

4.2.4 Growth analysis of *D. radiodurans* ΔRM strains

Physiological analysis of *D. radiodurans* ΔRM strains was performed by testing their growth in liquid TGY media. The growth phenotype of *D. radiodurans* ΔRM strains compared to wild type revealed no significant difference based on the growth curve, endpoint density or calculated growth rates (Figure 4-9A,B). This suggests that removal

A

Dilution	Nonselective		Selective (Nm5)	
	Blue	Pink	Blue	Pink
10^{-2}	TNTC	TNTC	366	4
10^{-3}	959	20	29	0
10^{-4}	104	0	4	0
Percent		**2.04%**		**1.08%**

B

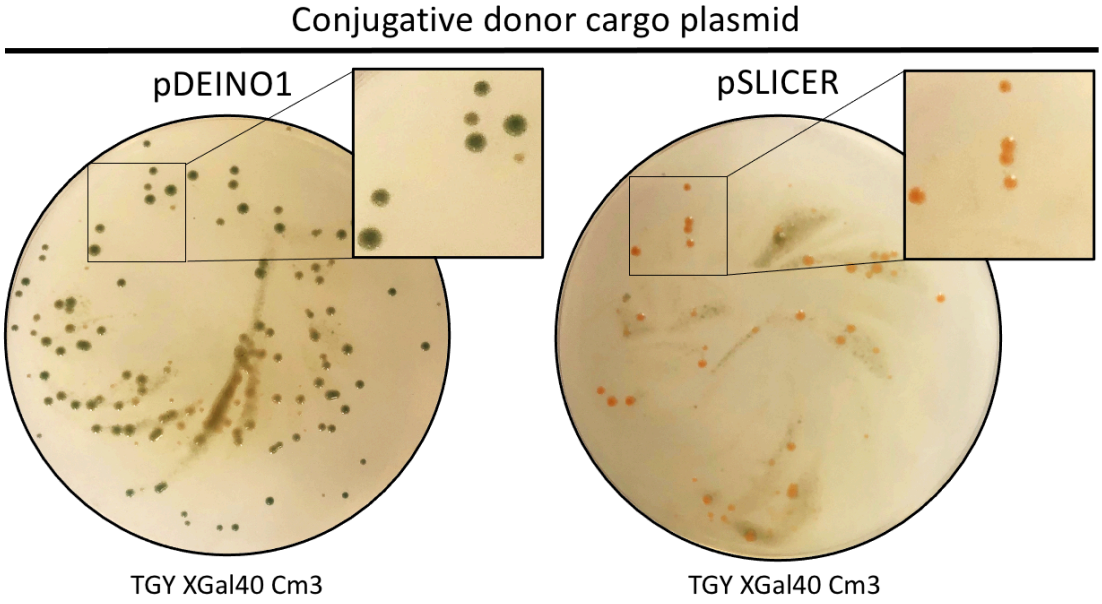
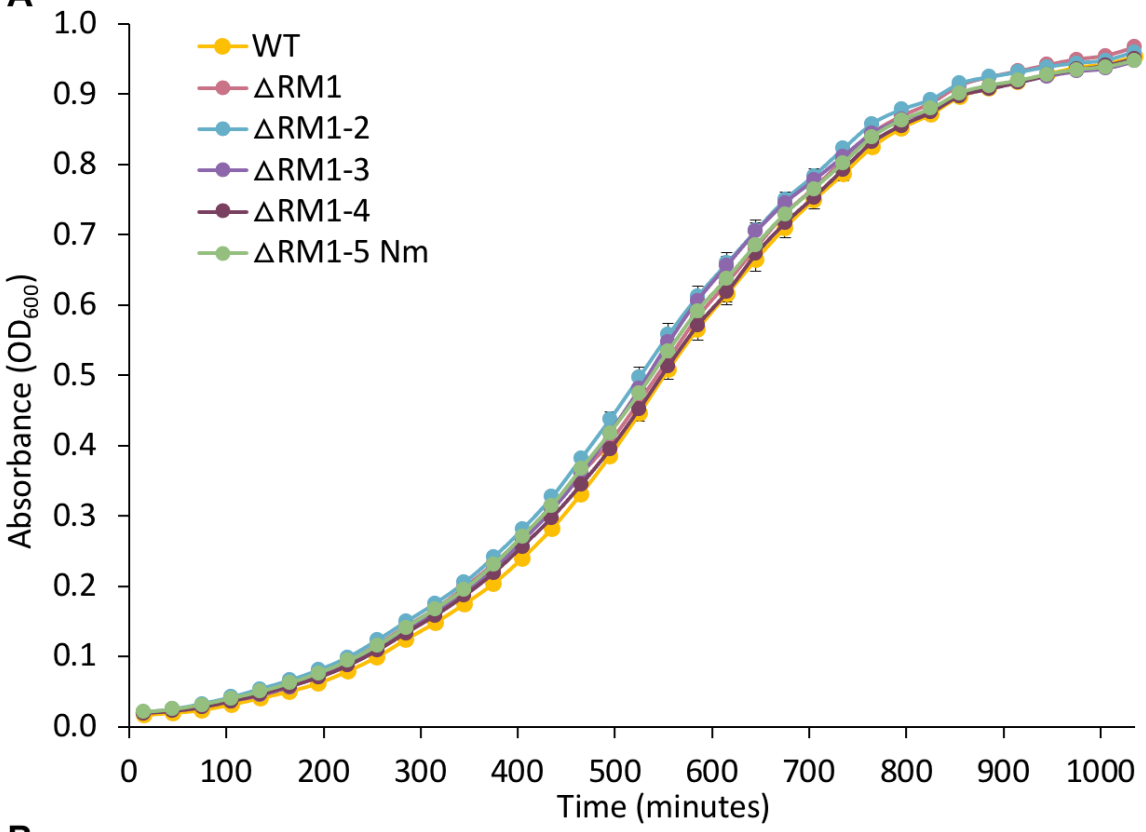


Figure 4-8. Validation of I-*Sce*I endonuclease function. (A) Serial dilutions of *D. radiodurans* ΔRM1-4 Nm^R plated on nonselective (TGY X-Gal) and selective (TGY X-Gal supplemented with neomycin) media. The number of blue and pink colonies was counted and the percentage of colonies that appeared pink over total colonies is reported. (B) Selective plates following conjugation of pDEINO1 and pSLICER from *E. coli* to *D. radiodurans* ΔRM1-4 Nm^R. Antibiotic concentrations are reported as μg mL^{-1}.

A



B

Strain	Mean Generation Time (min)	Standard Deviation (+/-)
WT	93	13
ΔRM1	98	13
ΔRM1-2	94	18
ΔRM1-3	91	13
ΔRM1-4	95	14
ΔRM1-5 Nm	94	16

Figure 4-9. Growth analysis of *D. radiodurans* seamless deletion strains. (A) Growth curves of *D. radiodurans* WT and ΔRM knockout strains grown in liquid TGY media for 17 hours. Each data point represents the mean of three biological and two technical replicates, with error bars representing standard error of the mean. (B) The average doubling time for WT *D. radiodurans* and ΔRM knockout strains grown in liquid TGY media is reported in minutes and represents the mean value of three biological and two technical replicates +/- the standard deviation.

of the first five R-M system genes did not result in any growth deficits in *D. radiodurans*, which is promising if this strain (or the full restriction minus strain) are to be used as synthetic biology chassis in the future.

4.2.5 Transformation and DNA assembly in *D. radiodurans* seamless deletion strains

Transformation of *D. radiodurans* ΔRM strains was performed using the ~6 kb pRAD1 plasmid to determine if these strains have improved transformation efficiency compared to wild type. Heat shock transformation was performed using 850 ng of plasmid DNA isolated from *E. coli* Epi300 into wild type and all five ΔRM strains (Figure 4-10). These results indicate that through the deletion of five R-M systems, we were able to improve transformation by approximately 3-fold from wild type to *D. radiodurans* ΔRM1-5 Nm^R with an average transformation efficiency of 3.86 x 10^1 and 1.27 x 10^2 CFU/μg DNA, respectively. This follows the pattern of similar studies that have been conducted to improve transformation efficiency of microbes such as the deletion of restriction endonucleases in *Mycoplasma mycoides* (24). Only a slight improvement in transformation efficiency was seen by removing a subset of the restriction endonucleases, and it is only by the removal of the final restriction endonuclease that a vast improvement was seen (24). We hypothesize that seamless removal of the final R-M system genes in *D. radiodurans* will result in a similar increase in transformation efficiency.

Although transformation to *D. radiodurans* was not largely improved, we tested *in vivo* DNA assembly in wild type and ΔRM1-5 Nm^R. We PCR amplified pRAD1 in two fragments with either 100, 500 or 1000 bp overlaps. Using our standard heat shock method, we transformed each cell type with ~1 μg of each PCR fragment and selected for transformants on TGY media supplemented with chloramphenicol. We did not observe any colonies from the assembly attempts in wild type or ΔRM1-5 Nm^R, indicating that the assembly was unsuccessful (data not shown). We hypothesize that DNA assembly will be improved in a fully restriction minus strain, allowing for improved uptake of

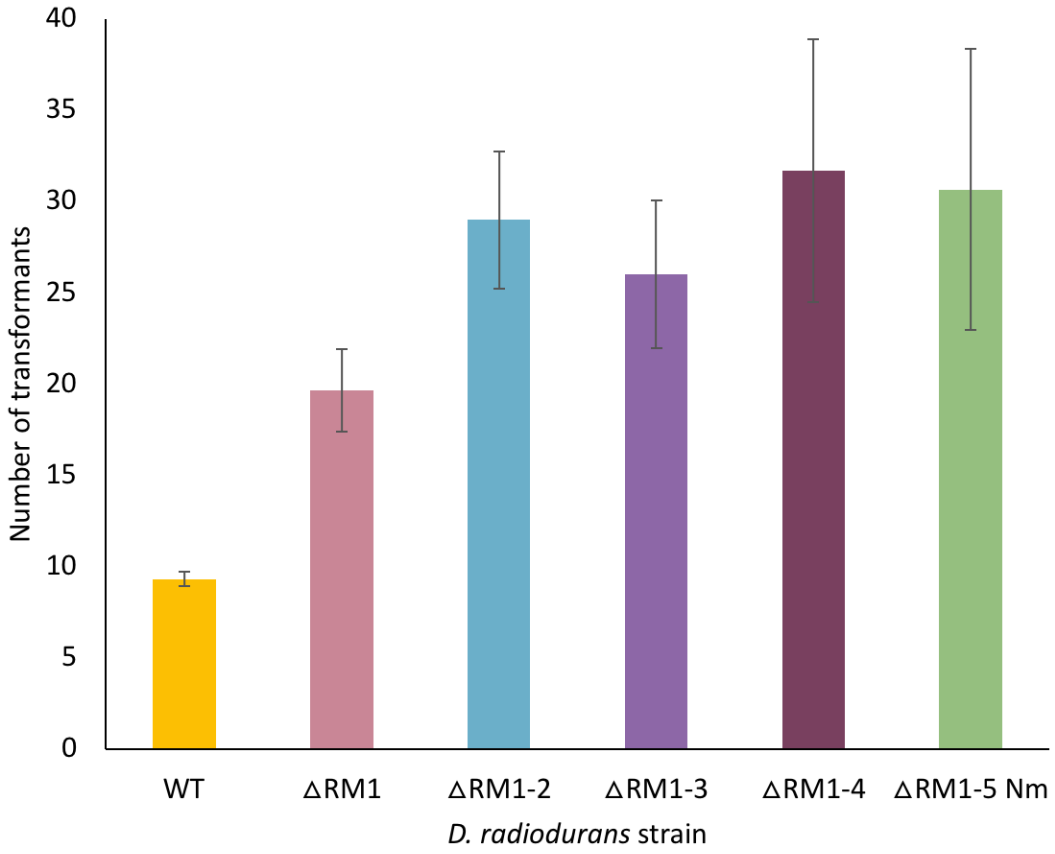


Figure 4-10. Transformation efficiency and growth analysis of *D. radiodurans* seamless deletion strains. Heat shock transformation of the pRAD1 plasmid to *D. radiodurans* WT and ΔRM knockout strains. The same amount of DNA was used for each transformation (850 ng) and the number of *D. radiodurans* transformants that grew on TGY supplemented with chloramphenicol 3 μg mL^{-1} is reported. Data is presented as a bar graph indicating the mean of three biological replicates with error bars representing standard error of the mean.

multiple linear DNA fragments. In addition, strategies to optimize assembly could be investigated such as spheroplasting, which is used for yeast assembly (8).

4.2.6 Conclusions

In summary, we have created the first seamless gene deletion strategy for *D. radiodurans* and demonstrated that SLICER can be used for the sequential deletion of endogenous genes. Using this seamless deletion method, homozygous insertions and deletions can be made rapidly across all copies of the *D. radiodurans* genome, and it is the first report of the *I-Sce*I endonuclease being used in this bacteria. We used the SLICER method to create a *D. radiodurans* strain with four restriction-system genes seamlessly deleted, and a fifth gene replaced with a selective marker. Physiological analysis of these strains showed no growth deficit and improved transformation efficiency. We believe the SLICER method will be invaluable for *D. radiodurans* engineering and allow for the seamless deletion of any DNA target of interest in the future, including the remaining R-M systems. The deletion of five of the six known restriction-systems in *D. radiodurans* is a big step towards the creation of a fully restriction minus strain, which we hypothesize will significantly improve transformation of DNA to *D. radiodurans*. The development of a restriction minus strain will vastly expand the synthetic biology applications of *D. radiodurans* as a host for DNA assembly and may allow for genome reduction or replacement for the study of extremophile biology.

4.3 Methods

4.3.1 Microbial strains and growth conditions

Deinococcus radiodurans R1 was grown at 30°C in TGY medium (5 g L^{-1} tryptone, 3 g L^{-1} yeast extract, 1 g L^{-1} potassium phosphate dibasic, and 2.5 mL of 40% w/v glucose) supplemented with appropriate antibiotics (chloramphenicol, 5 μg mL^{-1}; neomycin, 5 μg

mL^{-1}). *Escherichia coli* (Epi300, Lucigen) was grown at 37°C in Luria Broth (LB) supplemented with chloramphenicol, 15 μg mL^{-1}. *Escherichia coli* ECGE101 (Δ*dapA*) (25) was grown at 37°C in LB media supplemented with DAP, 60 μg mL^{-1}, and appropriate antibiotics (chloramphenicol, 15 μg mL^{-1}; gentamicin, 40 μg mL^{-1}). *Saccharomyces cerevisiae* VL6-48 (ATCC MYA-3666: MATα *his3*-Δ200 *trp1*-Δ1 *ura3*–52 *lys2ade2*–1 *met14 cir*^{0}) was grown at 30°C in 2X YPAD rich medium (20 g L^{-1} yeast extract, 40 g L^{-1} peptone, 40 g L^{-1} glucose, and 80 mg L^{-1} adenine hemisulfate), or in complete minimal medium lacking histidine supplemented with 60 mg L^{-1} adenine sulfate (Teknova Inc.) with 1 M sorbitol. All strains created in this study are summarized in Supplemental Table D-1.

4.3.2 Plasmid design and construction

All plasmids in this study (Supplemental Table D-2) were constructed from PCR amplified DNA fragments assembled using a yeast spheroplast transformation method as previously described(26). The primers used to amplify the fragments for plasmid assembly (Supplemental Table D-3) contained 20 bp binding and 40 bp of overlapping homology to the adjacent DNA fragment. Following assembly, DNA was isolated from *S. cerevisiae* and the plasmid pool was electroporated into *E. coli* Epi300. Plasmids from individual colonies were screened for correct assembly using multiplex PCR and diagnostic restriction digest. All plasmids were built to contain a pCC1BAC-yeast backbone allowing replication and selection in *E. coli* (chloramphenicol) and *S. cerevisiae* (-HIS) with a low-copy *E. coli* origin that can be induced to high copy with arabinose. They also have an origin of transfer (*oriT*) necessary for conjugation. pSD1-4: nonreplicating plasmids containing two ~1 kb regions of homology flanking ORF14075, *Mrr*, ORF15360, and *Mrr2*, respectively, amplified from wild-type *D. radiodurans* genomic DNA. Between the homology regions on the plasmids is an I-*Sce*I recognition site, a selective marker (*nptII*) and visual screening marker (*lacZ*) amplified from pDEINO1 and pET-24α(+)-lacZ, respectively, and an 80 bp duplication of the 3' end of homology region 1. The aforementioned elements make up the SD cassette. These plasmids also contain a second selective marker for *D. radiodurans* outside of the SD

cassette, *tetR/A* or *aadA1* amplified from pDEINO3 and pDEINO4, respectively (10). pSLICER: replicating plasmid built to contain a *D. radiodurans* codon-optimized *cat* gene under the control of a constitutive promoter (drKatA) and origin of replication amplified from pDEINO1 (10). A synthesized *D. radiodurans* codon-optimized I-*Sce*I endonuclease gene was also incorporated on this plasmid under the control of the PDR_2508 promoter and terminator set (23).

4.3.3 $CaCl_2$ transformation of *D. radiodurans*

For competent cells: A 50 mL culture of *D. radiodurans* was grown to an OD_{600} of 0.2. The culture was transferred to a 50 mL falcon tube and centrifuged at 3000 *g* for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in 250 µL of ice-cold 0.1 M $CaCl_2$ 15% glycerol solution using gentle agitation. The competent cells were aliquoted in 50 µL increments, frozen in a -80°C ethanol bath and stored at -80°C.

For transformation: 50 µL of competent cells per reaction were thawed on ice for 15 min. Then, 5 µL of transforming DNA (linear PCR product or plasmid) was mixed with the competent cells. The mixture was incubated on ice for 30 min then heat shocked in a 42°C water bath for 45 seconds. The tubes were returned to ice for 1 min and 1 mL of 2X TGY media was added to each tube. The recovery cultures were transferred to a 50 mL falcon tube and grown with shaking at 30°C for 2 hours at 225 rpm. Finally, 300 µL of the transformation mixture was plated on TGY media with appropriate supplements (chloramphenicol 3 µg mL^{-1} or neomycin 5 µg mL^{-1} and/or X-Gal 40 µg mL^{-1}) and incubated at 30°C for 2-3 days. Colonies were counted manually.

4.3.4 Conjugation from *E. coli* to *D. radiodurans*

Conjugation from *E. coli* to *D. radiodurans* was performed as previously described (10), with the following modifications. The donor strain was *E. coli* ECGE101 Δ*dapA* (27) harbouring pTA-Mob (28) and pSLICER. The *D. radiodurans* R1 recipient strains with the integrated SD cassettes were grown in TGY media supplemented with neomycin (5

μg mL^{-1}) prior to conjugation. The selective plates were TGY media supplemented with chloramphenicol (3 μg mL^{-1}).

4.3.5 *D. radiodurans* genomic DNA isolation

Alkaline lysis was performed using 3 mL of saturated culture as previously described (26) to extract *D. radiodurans* genomic DNA for analysis.

4.3.6 Multiplex PCR analysis of *D. radiodurans* knockouts

Multiplex PCR analysis was performed according to the manufacturer's instructions for "Standard Multiplex PCR" (Qiagen Multiplex PCR Handbook) with the following modifications and the primers listed in Supplementary Table D-3. A final volume of 20 μL was used and reaction mix components were adjusted accordingly. A volume of 1 μL of undiluted template DNA and 1 μL of dimethyl sulfoxide (DMSO) was used in the reaction mix. Thermocycler conditions were chosen according to the "Universal Multiplex Cycling Protocol" with the initial activation step decreased to 5 min, using an annealing temperature of 60°C, and 30 cycles. Gel electrophoresis was used to visualized 2 μL of the PCR product on a 2% agarose gel.

4.3.7 Spot plating *D. radiodurans*

D. radiodurans was grown overnight in 5 mL cultures of TGY media supplemented with the appropriate antibiotics (none, neomycin or chloramphenicol). The cultures were diluted to an OD_{600} of 0.1 before performing 10-fold serial dilutions in TGY media up to 10^{-5} dilution. Then, 5 μL of each dilution was plated on nonselective TGY media and/or TGY media supplemented with appropriate antibiotics and incubated at 30°C for 2-3 days.

4.3.8 *D. radiodurans* growth curve and doubling time calculation

Growth rates were evaluated for *D. radiodurans* strains: wild type, ΔRM1, ΔRM1-2, ΔRM1-3, ΔRM1-4, and ΔRM1-5 Nm^R. Single colonies were inoculated into 5 mL of liquid TGY media and grown overnight at 30°C with shaking at 225 rpm. 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, along with a TGY media only control. In the Epoch 2 (BioTek, USA) plate reader, strains were grown at 30°C with continuous, orbital shaking (559 cpm). Absorbance (A_{600}) measurements were taken every 15 min for 24 h for a total of 97 readings using Gen5 data analysis software version 3.08.01 (Biotek, USA). This experiment was performed with three biological replicates, each with two technical replicates. Growth curves were plotted with data points representing the average of six measurements for each strain with error bars representing standard error of the mean. For simplicity, every other time point was omitted; therefore, readings are presented for every 30 min and the curve is cut off at the 17 hour time point when cultures approached end point density. The doubling time of each replicate was determined using the R package Growthcurver (Sprouffske K., Growthcurver, http://github.com/sprouffske/growthcurver, 2016) (29). The doubling time is reported as an average of the six replicates for each strain, and the standard deviation was calculated.

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Chapter 5

5 General Discussion

5.1 Bacterial chassis for synthetic biology applications: *S. meliloti* and *D. radiodurans*

Microorganisms possess attractive genetic, metabolic and physiological traits that can be exploited for synthetic biology applications to address global problems in agriculture, health, manufacturing, environmental pollution, and sustainability (1). *Escherichia coli* and *Saccharomyces cerevisiae* are traditional chassis that are most frequently used for these purposes due to their well-characterized genomes, physiology, and extensive genetic toolkits (2). Many other physiologically attractive microbes exist but are often underutilized in biotechnology due to a lack of genetic characterization and tools required to engineer strains, explore their capabilities, and optimize performance.

Sinorhizobium meliloti and *Deinococcus radiodurans* are two bacterial chassis that have great potential to address some of these global challenges and advance synthetic biology research. The symbiotic nitrogen-fixing bacteria, *S. meliloti*, is a promising chassis for agricultural applications, vitamin and biomaterial synthesis (3–5). *S. meliloti* is particularly of interest as a commercial agricultural chassis organism as its use could avoid infringing on patents for *Agrobacterium*-mediated transformation for plant engineering and crop improvement (6). *D. radiodurans* is a polyextremophile and a promising bacterial chassis for bioremediation, biopharmaceuticals, and aerospace applications (7). Both organisms have GC-rich, multipartite genomes and unique metabolic or physiological properties that may allow them to outperform traditional chassis for niche applications (8,9). Increasing the number and variety of available genetic tools in these species could enhance application-based engineering, improve their utility as laboratory chassis, and elucidate foundational knowledge of their biology. Therefore, the goal of this thesis has been to expand the genetic toolkits of *S. meliloti* and *D. radiodurans* to facilitate strain engineering for synthetic biology applications.

5.2 Genome reduction improves chassis utility

Genome reduction can streamline engineering and improve the utility of a chassis for bioproduction by providing a simplified genetic background (10). Bacteria with multipartite genomes, like *S. meliloti* and *D. radiodurans*, may be of particular interest for genome reduction as these strains contain segmented genomes with one or more chromosomes as well as secondary replicon(s). These replicons are typically large, naturally occurring plasmids (*i.e.*, megaplasmids) that contain genes required for niche-specific or specialized functions, such as the ability to utilize certain carbon sources (11).

In the case of *S. meliloti*, several genome-reduced strains have been created, lacking either or both of its secondary replicons: the pSymA megaplasmid and pSymB chromid (11). With these large (> 1 Mb) replicons removed, the endogenous *repABC*-type origins of replication were available for use in synthetic plasmids. In addition to streamlining the *S. meliloti* genome, genome deconstruction was used to identify the minimal set of genes required for nitrogen-fixing symbiosis with the legume *Medicago sativa.* Researchers determined that 58 genes, all localized to the pSymA megaplasmid, were required to sustain symbiotic nitrogen fixation (12). Reintroduction of this minimal gene set into the genome-reduced strains of *S. meliloti* described in this thesis may re-establish their symbiotic capabilities for agricultural applications.

Conversely, there have been no attempts to significantly reduce the genome of *D. radiodurans* thus far, likely due to a lack of large-scale genome engineering tools. Using the genome reduction of *S. melilot* as a guide, it is reasonable to surmise that the secondary replicons of *D. radiodurans* (*i.e.*, MP1 megaplasmid, CP1 plasmid) may pose a promising starting point for genome reduction. Genome deconstruction could reveal genetic contributions to specialized functions in *D. radiodurans* including its extreme stress tolerance.

5.3 Plasmid diversity is essential for synthetic biology applications

Plasmids are a valuable tool and platform for cloning, selection, protein expression, strain engineering, and so on. Shuttle plasmids are particularly useful for synthetic biology as they can replicate in multiple host species, thereby allowing for assembly or manipulation in one microbe followed by transfer to another (13). Versatile MHS plasmids (*i.e.*, pAGE, pBGE) were created for *S. meliloti* with modular components including the *repABC*-type origins from the pSymA and pSymB replicons. These plasmids contain an origin of transfer (*oriT*) for conjugation and were stably propagated in the *S. meliloti* genome-reduced strains as well as *E. coli*, yeast, and microalgae (14). For *D. radiodurans*, replicative and non-replicative MHS plasmids (*i.e.*, pDEINO) were modified from the pAGE plasmids to include an origin of replication and/or various selectable markers for *D. radiodurans* (15).

These MHS vectors will be useful as a platform for engineering and biosynthesis in both *S. meliloti* and *D. radiodurans* since they can be transferred to a wide range of model organisms via conjugation and can be maintained in hosts at different copy number. In *S. meliloti* and *D. radiodurans,* the plasmids are maintained at a low copy number but can be induced to high copy in *E. coli.* High copy plasmids allow for high gene expression levels and/or increased plasmid concentration for DNA isolation. However, constitutively high expression can lead to metabolic burden on the host organism and potentially plasmid instability (16). Low copy plasmids, that maintain chromosome-level copies, may overcome these difficulties by decreasing protein expression and minimizing metabolic burden. In practice, use of low copy plasmids has increased plasmid stability, particularly when maintaining large DNA constructs (16). Therefore, it is often advantageous for synthetic plasmids to have the capacity for both low and high copy number.

5.4 Restriction-modification systems impede chassis engineering

Restriction-modification (R-M) systems are a hindrance to the domestication of non-model organisms, preventing efficient transformation (17). The *S. meliloti hsdR* gene of the Type I R-M system gene cluster (*i.e.*, *hsdRSM*) restricts foreign DNA uptake; previously, deletion of this gene or gene cluster in wild type *S. meliloti* resulted in increased transformation efficiency (18,19). Therefore, to further the utility of *S. meliloti* as a bacterial chassis and ensure high transformability, *hsdR* knockouts in the three genome-reduced strains (lacking either or both pSymA and pSymB) were created (14). Transformation of MHS plasmids was then demonstrated to be successful to *S. meliloti* ΔpSymA Δ*hsdR* through electroporation and a novel PEG-mediated transfer method.

Unlike the single knockout required for *S. meliloti*, *D. radiodurans* has six putative R-M systems (20). As such, the transformation of episomes is inefficient, particularly those isolated from different species. To improve transformation of *D. radiodurans*, methods for the transfer of large plasmids and for the creation of multiple knockouts in a single *D. radiodurans* strain had to be developed (discussed in section 5.5.2 and 5.6.2, respectively).

5.5 Conjugation is versatile method of DNA transfer

Conjugation is one of three main mechanisms of horizontal gene transfer in bacteria that can be harnessed in the laboratory as a DNA delivery method (21,22). The conjugative donor bacteria carries a conjugative plasmid, in this case the RP4/RK2 derivative pTA-Mob (21), and a cargo plasmid with an origin of transfer (*oriT*), like our MHS vectors. The conjugative machinery is needed to form a pilus between the donor and recipient organisms, recognize and nick the *oriT*, and mobilize the cargo plasmid to the recipient cell (23). Conjugation is a versatile method of DNA transfer that has been previously demonstrated from *E. coli* to a variety of recipient organisms including Gram-negative and Gram-positive bacteria, as well as eukaryotic organisms such as yeast, microalgae, and mammalian (human) cells (23–27). Thus, conjugation is a broadly applicable method

of transformation that could be an attractive alternative to standard transformation methods for organisms that are difficult to transform. Additionally, conjugation complements current transformation methods by allowing plasmid propagation or bacterial targeting through microbial communities or biofilms *in situ* (in natural microbial communities) (28).

5.5.1 Transfer of large-scale DNA

Conjugation has been demonstrated as an efficient method for the transfer of large DNA constructs including whole genomes greater than 1 Mb in size (29,30). Unlike using transformation to deliver linear DNA or episomes, conjugation does not require any competent cell treatment, DNA extraction, or PCR amplification as it allows for DNA transfer directly from donor to recipient cells. Due to the mechanism of conjugation, plasmids introduced via this method are resistant to degradation by native restriction-systems that target double-stranded DNA. This may allow for large plasmids or whole chromosomes to be delivered to strains with active restriction-systems more easily than other DNA delivery methods (17).

5.5.2 Interspecies and interkingdom DNA transfer

The pAGE MHS plasmid, harbouring the pSymA origin of replication, was shown to be efficiently introduced into *S. meliloti* ΔpSymA Δ*hsdR* through direct conjugation from an *E. coli* Δ*dapA* donor strain harbouring the pTA-Mob conjugative helper plasmid (14). The same *E. coli* donor was used to demonstrate conjugation of the pDEINO1 plasmid to *D. radiodurans* as a new method of DNA delivery to this chassis (15). The use of a Δ*dapA* conjugative donor has been demonstrated in a previous study (31) and proves to be an effective strategy for counterselection against a donor bacteria. This strategy circumvents the use of antibiotics for counterselection and is particularly useful for *D. radiodurans* which is sensitive to very low concentrations of antibiotics.

The conjugation frequency of plasmids transferred from *E. coli* to *S. meliloti* and *D. radiodurans* recipients were on the order of 10^{-1} and 10^{-5}, respectively (14,15). The observed decrease in conjugation frequency with *D. radiodurans* as a recipient could be attributed to the mechanistic differences between Gram-positive and Gram-negative conjugation, as Gram-positive bacteria facilitate contact between donor and recipient cells through surface adhesins rather than conjugative pili (32). It could also be due to the complex cell envelope of *D. radiodurans* or the presence of active R-M systems (33,34). Gram-negative to Gram-positive conjugation is not as commonly reported and any additional examples of this will help to elucidate this mechanism of DNA transfer and contribute to the foundational knowledge of conjugation, which is still being investigated.

Conjugation from *E. coli* to *S. meliloti* strains has been demonstrated previously through triparental mating experiments, however these often employ *E. coli* S17-1-mediated conjugal transfer (19,35,36). *E. coli* S17-1 is a donor strain with RP4 conjugative machinery integrated into its chromosome. Although this strain is extensively used, several studies have reported issues caused by plasmid modifications of unknown origins, as well as mobilization of an active bacteriophage Mu genome or *E. coli* chromosomal DNA to the recipient (21). The use of a broad-host-range helper plasmid, pTA-Mob, has not only been proposed to circumvent these problems, but it could be used to turn other bacteria into conjugative donors (21).

S. meliloti ΔpSymA Δ*hsdR* was equipped with conjugative machinery by transforming it with pTA-Mob (21). *S. meliloti* is an attractive conjugative donor because it has a high G+C-content genome, a slower growth rate to prevent outcompeting recipient cells during conjugation, and could be used for agriculture-based applications. We demonstrated the ability of *S. meliloti* to transfer the MHS plasmids via conjugation to several bacterial and eukaryotic model organisms across kingdoms including *E. coli*, *S. cerevisiae* and *P. tricornutum*. Conjugation frequency to bacterial and eukaryotic recipients was found to be on the order of 10^{-1} and 10^{-6}, respectively, with no substantial mutations introduced (14). Significantly, the demonstration of *S. meliloti* as a novel conjugative donor to eukaryotes can be added to the body of work investigating bacterial conjugation to eukaryotic cells (23,26,27). The conjugative strains and plasmids

developed here will complement other conjugation-based tools that have been established for *S. meliloti,* including *oriT*-directed cloning for the manipulation of large genomic DNA fragments (37).

5.6 Conjugation as a tool for engineering non-traditional chassis

Conjugation can be a useful tool for synthetic biology research as well as industrial applications and is a particularly attractive method to streamline the workflow for genetic manipulation of organisms. For example, plasmids and pathways can be assembled and manipulated *in vitro* (or *in vivo* in the donor strain), introduced into a conjugative donor strain, and directly transferred to the target organism of interest (23). This eliminates the need for high-yield plasmid DNA extraction or expensive equipment such as a gene gun for biolistic transformation. Thus, conjugation is a promiscuous method of DNA transfer and can be used as a method to engineer specific target cells.

5.6.1 Genetic modifications

Homologous recombination-based genetic tools have been used previously to engineer a variety of non-model microbes, including *D. radiodurans* (38–40). However, these methods relied on amplification of PCR products or transformation of nonreplicating plasmids (41), which was shown to be inefficient for introducing large (>20 kb) plasmids (15). To enable precise genome modifications, a conjugation-based system for gene replacement was designed utilizing homologous recombination of a selectable marker in *D. radiodurans*. This system was used to knock out several R-M system genes in *D. radiodurans* to produce single and double knockout strains (15). We demonstrated that this method could enable gene deletions across all genomic copies of *D. radiodurans,* with no off-target modifications, and that the nonreplicating plasmids were quickly lost following recombination. Nanopore sequencing used in this study allowed the acquisition of long reads to resolve the wild type genome sequence of *D. radiodurans* (which

contradicts the most recent PacBio sequence reported), and confirm gene deletions in the double knockout strain (15). This conjugation-based gene deletion method can be modified with other selective or screening markers to target any DNA sequence of interest and will be valuable for creating *D. radiodurans* mutants for synthetic biology and studying biological processes.

5.6.2 Seamless gene deletions

Transformation and maintenance of synthetic constructs is most commonly achieved in *D. radiodurans* using antibiotics and their respective resistance gene. However, to make multiple knockouts in a single strain, it would be beneficial to have a method to recover selectable markers for reuse. Recently, a Cre-lox system was developed to allow for removal of integrated selectable markers (42). However, this method leaves behind loxP sites; therefore, there is currently no method for creating seamless gene deletions in this bacteria. To address this, a robust system for creating seamless gene deletions in *D. radiodurans* was developed entitled Seamless Loss of Integrated Cassettes using Endonuclease cleavage and Recombination (SLICER).

This method was demonstrated successfully for the sequential deletion of four R-M system genes. Rapid gene deletions were observed across all genomic copies of *D. radiodurans* using this method (after a single passage on selective media) compared to previous methods that reported the need to subculture for 30-35 passages alternating growth in liquid and solid media to obtain homozygous gene knockouts (40). Physiological analyses of these R-M knockout strains showed no significant differences in growth rate compared to wild type, and improved transformation efficiency of a small plasmid (~6 kb). The tools developed here will allow for the deletion of the remaining restriction endonucleases and other endogenous *D. radiodurans* proteins of interest such as those involved in the biosynthesis of essential amino acids for the generation of auxotrophic strains, or proteases in production strains.

5.6.3 Whole genome engineering

Synthetic biologists are interested in the cloning, manipulation and delivery of whole chromosomes or large DNA fragments for the study of intractable organisms, genome reduction or synthesis, and reintroduction of a modified genome into original hosts (43). This thesis presents tools for large-scale genome manipulation which have yet to be developed for *D. radiodurans* that will complement existing engineering strategies and tools. Specifically, the current genetic tools do not provide a solution for the introduction of large-scale DNA into the cell (15). Using the developed conjugation method and MHS plasmids, the large, 178 kb MP1 megaplasmid from *D. radiodurans* was captured *in vivo* and cloned in *E. coli.* This is a foundational step towards genome reduction and further characterization of this secondary replicon. The ability to clone, recode, or minimize the *D. radiodurans* genome will help to expand its utility as a synthetic biology or industrial chassis.

With further testing, the developed MHS plasmids could provide a mechanism for the cloning of large-scale DNA and potentially whole chromosomes in *S. meliloti*. In the Karas laboratory, these plasmids have already been used for cloning whole mitochondrial genomes from microalgae in *E. coli* and *S. cerevisiae* (44,45), in the expansion of genetic toolkits for *Mesorhizobium* and *Bradyrhizobium* species, and are being adapted for the generation of nitrogen-fixing organelles from *Rhizobiales* in yeast (46).

5.7 Conclusions and future directions

To develop *S. meliloti* as an ideal chassis for synthetic biology applications, further strain and tool developments were required. This was accomplished through the creation of restriction-system minus strains (in genome-reduced backgrounds), MHS plasmids, methods for DNA uptake, and methods for DNA delivery to other bacterial and eukaryotic chassis organisms. This is the first report of i) a restriction minus, genome-reduced strain, ii) PEG-mediated transformation, and iii) conjugation from *S. meliloti* to eukaryotic recipients (*i.e.*, yeast and microalgae). The plasmids designed in this work address the need for shuttle plasmids that can be easily transferred between multiple

organisms, either bacteria or eukaryotes. The development of *S. meliloti* as a conjugative chassis for interkingdom DNA transfer may prove to be useful for the maintenance of high G+C-content DNA on these plasmids, and for the engineering or study of the demonstrated recipients or other target organisms such as agriculturally-relevant strains. Further characterization of the MHS vectors regarding their ability to clone DNA of varying sizes and G+C content should be performed to determine the limitations of these constructs. Although the *S. meliloti* strain lacking pSymB or both native replicons have slower growth rates compared to the ΔpSymA strain, these genome-reduced strains should be developed as conjugative hosts and could utilize the pBGE plasmids generated in this study. With the addition of the tools and strains developed in this work, the genetic toolkit for *S. meliloti* is well-established with genetic parts, plasmids, DNA delivery methods, restriction-system minus strains, and engineering strategies.

Compared to more well-studied bacterial chassis, the genetic toolkit for *D. radiodurans* is underdeveloped with limited genetic parts, plasmids, DNA delivery methods, and engineering strategies. To begin developing *D. radiodurans* as an ideal chassis for synthetic biology applications, further strain engineering and tool development was required. This was achieved by creating tools and methods for improved DNA uptake, genome engineering and whole chromosome cloning. This is the first report of i) conjugation as a DNA delivery method, ii) cloning of the MP1 megaplasmid in *E. coli*, iii) conjugation-based gene knockouts, iv) seamless gene deletions, and v) a strain with five of the six known R-M systems deleted. The constructed plasmids will be invaluable for future engineering efforts in *D. radiodurans* with the ability to propagate in several other model organisms. Conjugation was demonstrated as an efficient method to deliver large plasmids, and a useful strategy for whole chromosome cloning and homozygous gene knock out in *D. radiodurans*. Together, we believe these tools will enable small and large-scale genome engineering of *D. radiodurans*. Further biological characterization of the R-M knock out strains should be performed, including whole genome sequencing and methylation analysis. This could elucidate the methylation sites for the currently uncharacterized R-M systems. Upon creation of a full restriction minus strain, physiological analyses should be repeated to

determine if there are any detriments or improvements to growth or transformation efficiency. If transformation efficiency is improved, *in vivo* DNA assembly should be retested.

This thesis has aimed to improve *S. meliloti* and *D. radiodurans* as bacterial chassis through the development of tools and diverse plasmids to improve genome reduction, DNA delivery as well as small- and large-scale genome engineering. The tools and strains described here will significantly contribute to the future of *S. meliloti* and *D. radiodurans* research and their utility in the synthetic biology field. Prior to utilization of *S. meliloti* and *D. radiodurans* as bacterial chassis for applications outside of the laboratory, it will be important to implement biocontainment strategies in these organisms and plasmids. The expanded genetic toolkits developed here will advance the engineering capacity of *S. meliloti* and *D. radiodurans* chassis, and in turn, lead to further biological characterization, novel engineering technologies, and synthetic biology solutions. As Dr. Jay Keasling wrote, “while the development of biological components might be less “sexy” than the [direct] development of solutions to important problems, those components will enable many solutions, not just the ones for which the components were developed” (47).

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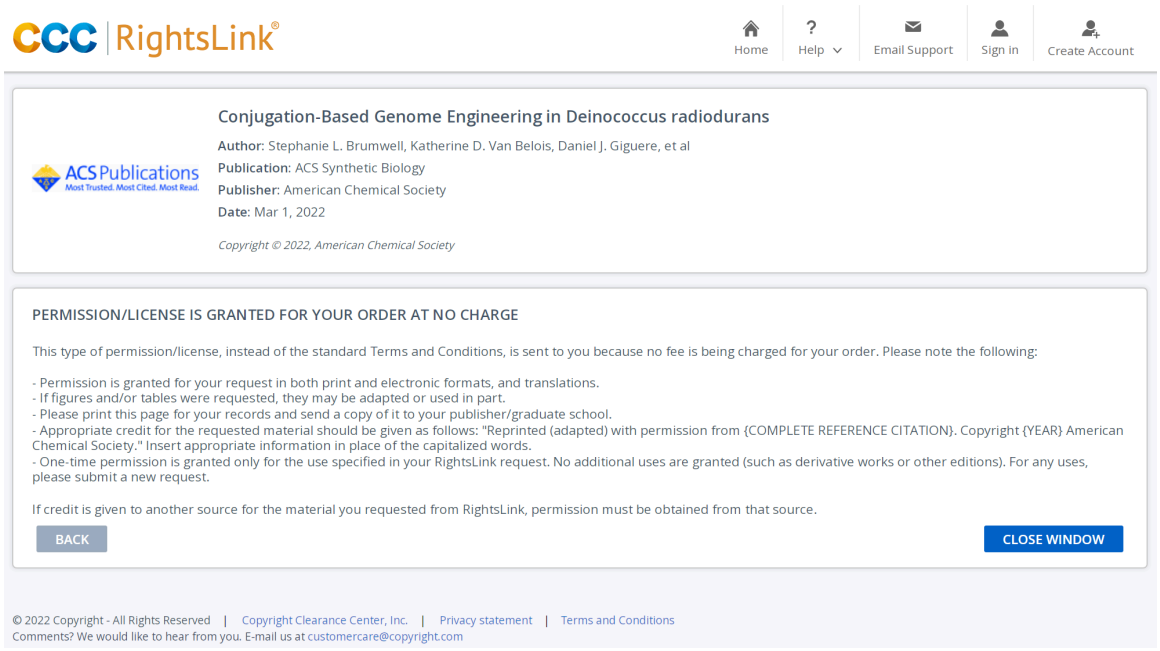

Appendix B: Supplemental Information for Chapter 2

B.1 Supplemental Figures

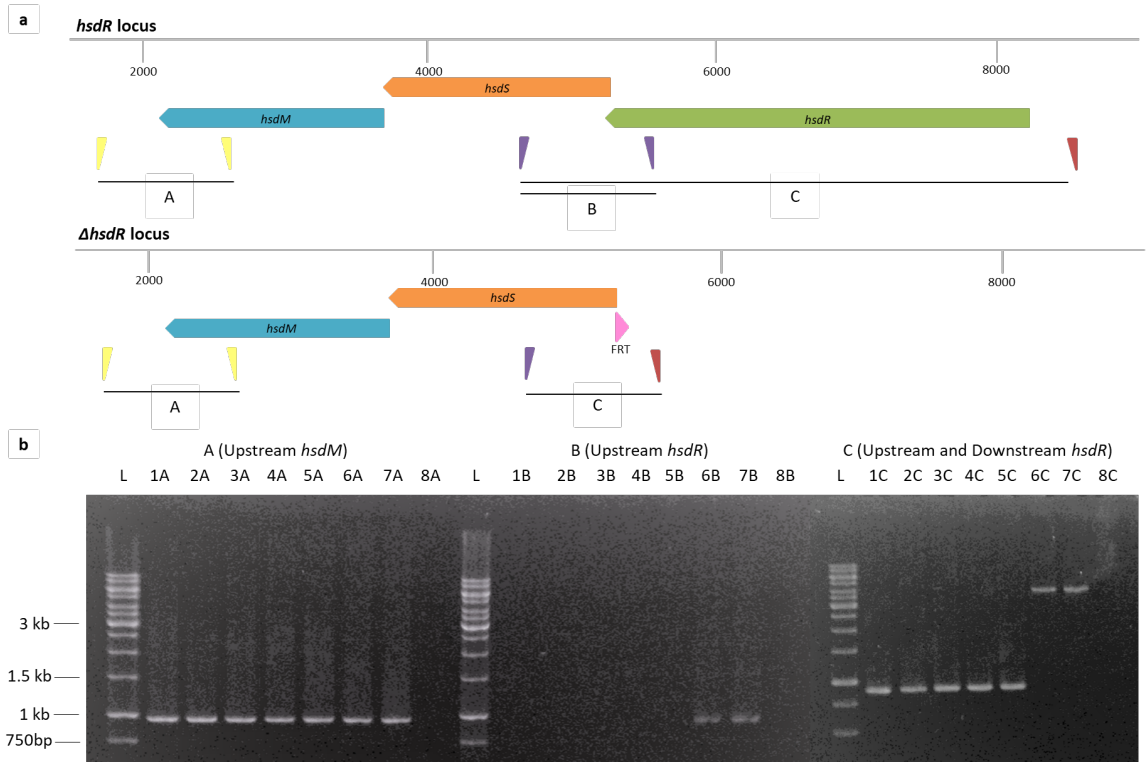

Figure B-1. Verification of *hsdR* deletion in reduced *S. meliloti* strains by diagnostic PCR. (a) Schematic of primer set locations (A, B and C) at the *hsdR* gene locus in wild-type *S. meliloti* when the *hsdR* gene is present (*hsdR* locus), and when the *hsdR* gene is replaced with FRT-Km/Nm-FRT cassette, which was subsequently excised via Flp recombinase (Δ*hsdR* locus). The pink arrow indicates an FRT site following loss of the FRT-Km/Nm-FRT cassette. (b) Gel electrophoresis of diagnostic colony PCR conducted with primer sets A, B, and C on wild type and designer *S. meliloti* strains. Expected band size for primer set A is 953 bp if the *hsdR* gene is present or absent. Expected band size for primer set B is 912 bp if the *hsdR* gene is present, and no band is expected if the *hsdR* gene is absent. Expected band size for primer set C is 4013 bp if the *hsdR* gene is present, and 787 bp if the *hsdR* gene is absent. L, 1 kb ladder. 1, RmP3909 ΔpSymAB Δ*hsdR*. 2, RmP3952 ΔpSymB Δ*hsdR*. 3, RmP3953 ΔpSymA Δ*hsdR*. 4, RmP3954 Δ*hsdR*. 5, Rm5000 ΔpSymA Δ*hsdR* Rif^R. 6, RmP110 wild type. 7, RmP110 wild type isolated gDNA. 8, no DNA control.

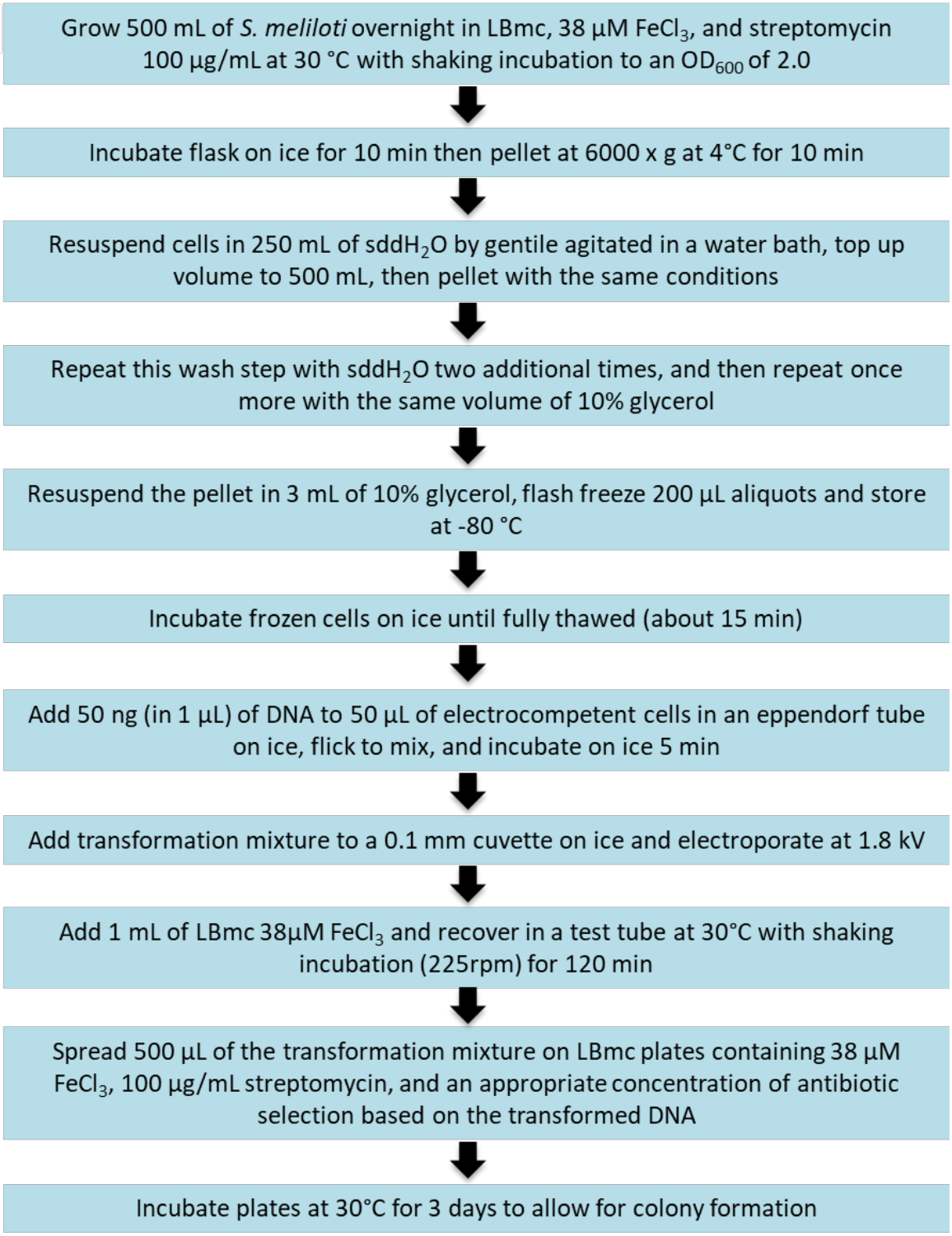

Figure B-2. Workflow of optimized electroporation transformation protocol for *S. meliloti*.

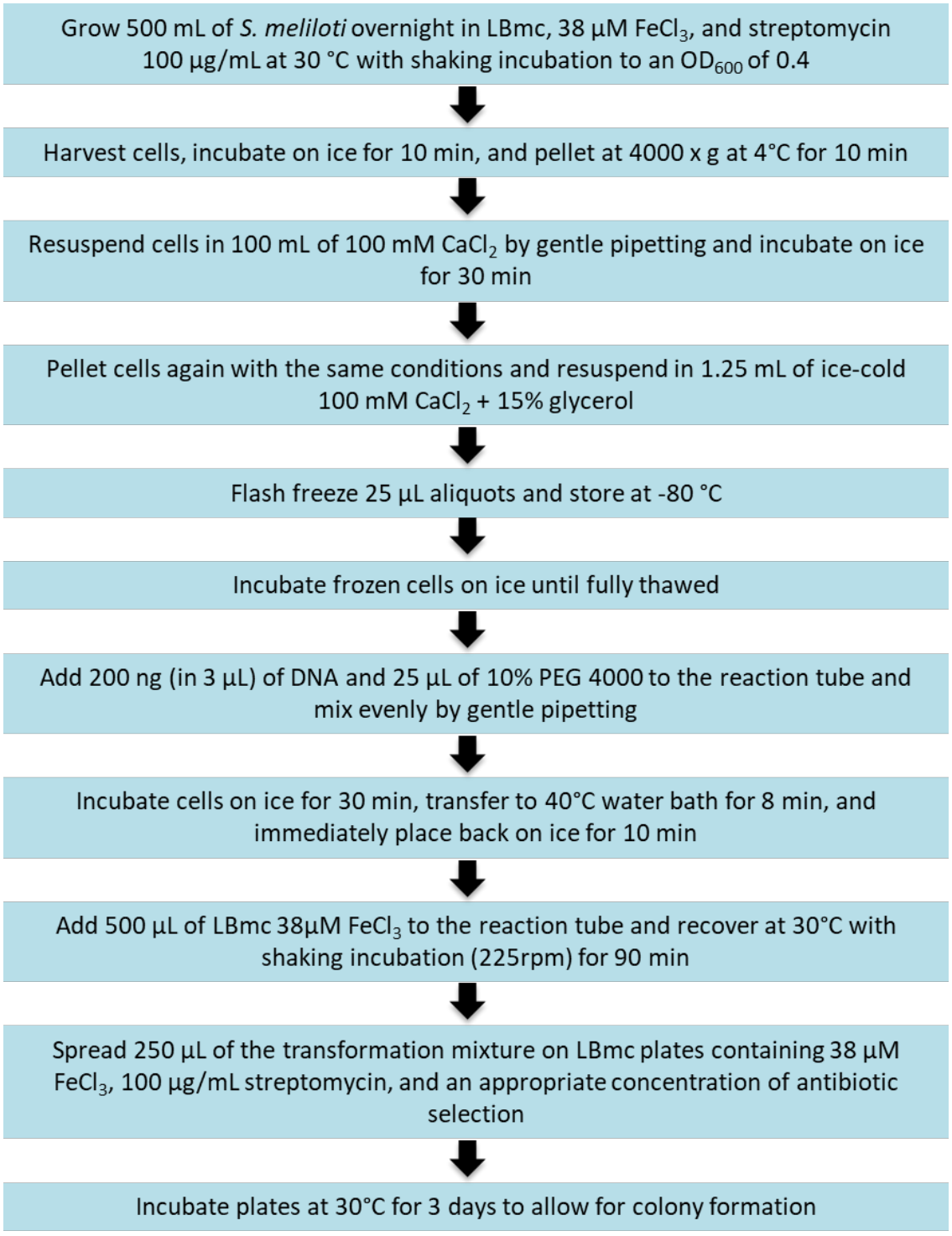

Figure B-3. Workflow of optimized PEG-mediated transformation protocol for *S. meliloti*.

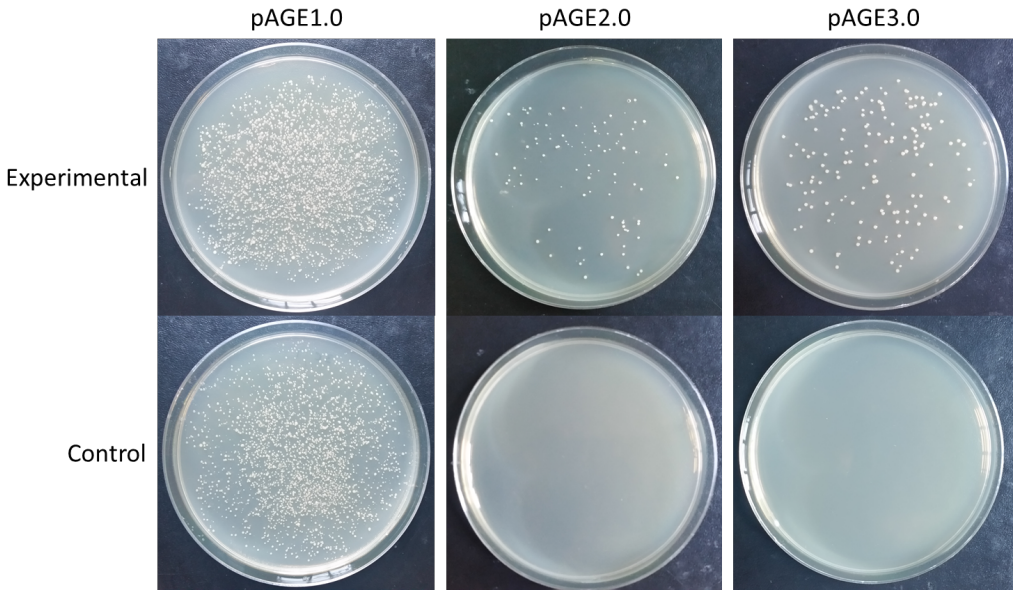

Figure B-4. PEG-mediated transformation of pAGE vectors into *S. meliloti*. Experimental and control plates from PEG-mediated transformation of pAGE1.0, pAGE2.0 and pAGE3.0 into *S. meliloti* RmP4122 ΔpSymA Δ*hsdR*. Control plates contain *S. meliloti* RmP4122 ΔpSymA Δ*hsdR* cells transformed with no DNA. pAGE1.0 transformation results were discarded due to the comparable number of colonies consistently observed on experimental and control plates.

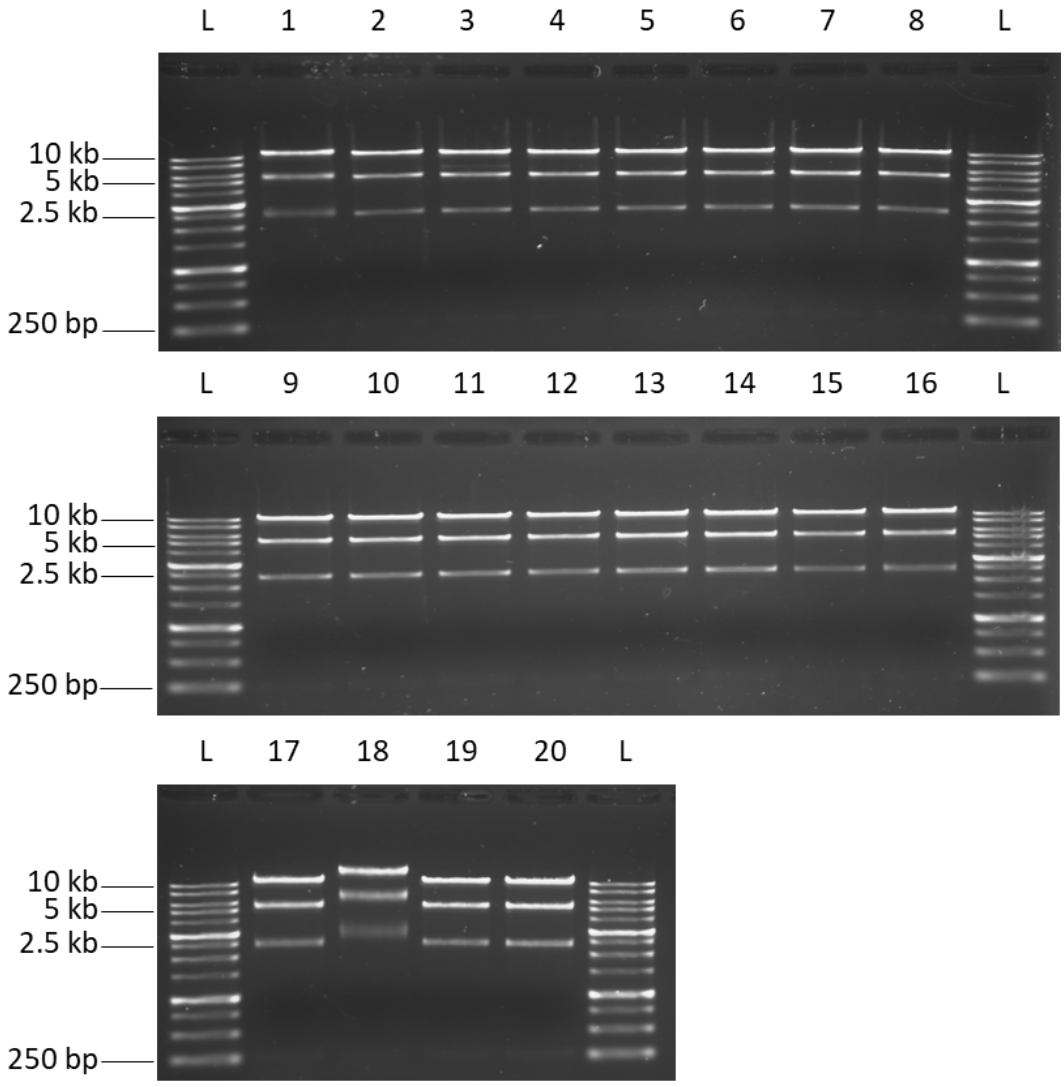

Figure B-5. **EcoRV-HF diagnostic digest of pAGE1.0 vectors extracted from 20 *E. coli* colonies following conjugation from *S. meliloti* to *E. coli*.** Expected band sizes of the pAGE1.0 vector following diagnostic restriction digest by EcoRV-HF are 10,288 bp, 5235 bp, 2377 bp, and 229 bp following gel electrophoresis on a 1% agarose gel. L, 1 kb ladder. 1-20, the 20 pAGE1.0 vectors extracted from *E. coli* and digested. Notes: 1) the 229 bp band is very faint and barely visible in this image; 2) clone number 3 (extra band between 10,288 bp and 5235 bp) and 18 showed incorrect digest pattern.

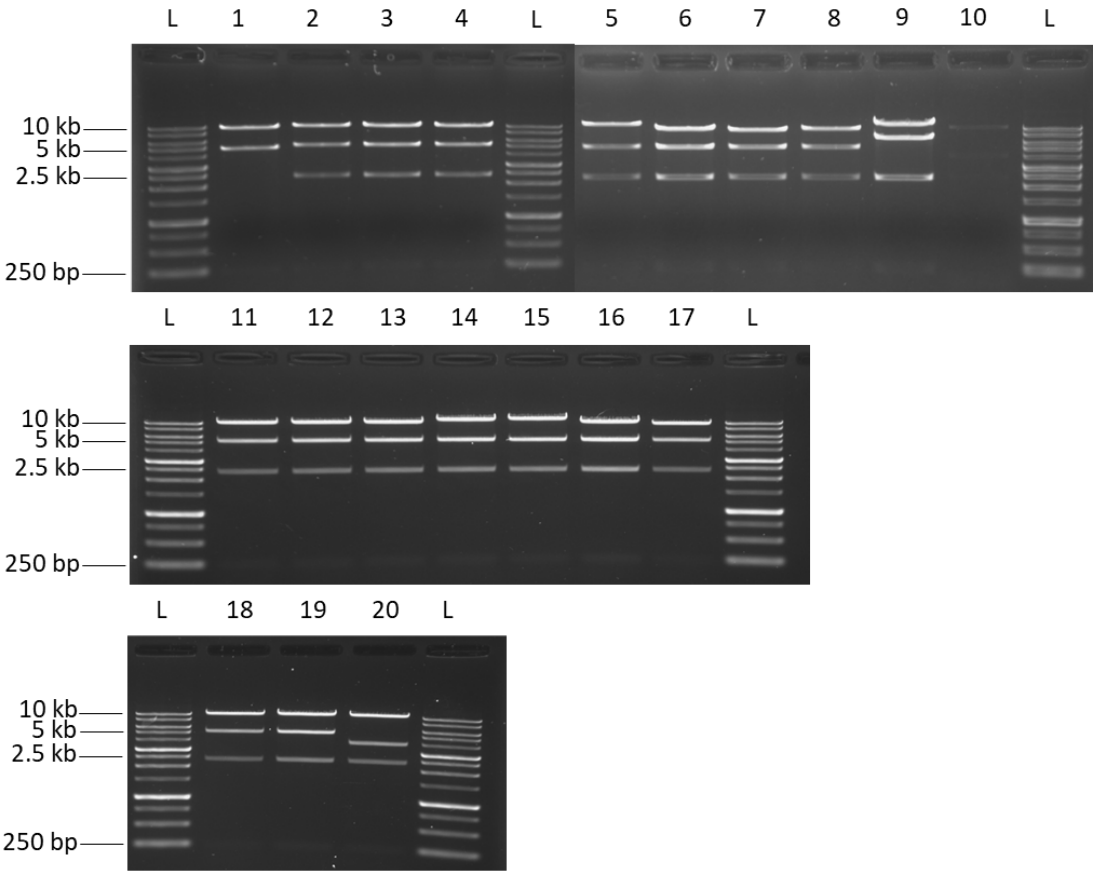

Figure B-6. **EcoRV-HF diagnostic digest of pAGE1.0 vectors extracted from 20 *E. coli* colonies following conjugation from *S. meliloti* to *P. tricornutum.*** Following conjugation, pAGE1.0 vectors were isolated from *P. tricornutum*, transformed into *E. coli*, induced to high copy and isolated again. Expected band sizes of the pAGE1.0 vector following diagnostic restriction digest by EcoRV-HF are 10,288 bp, 5235 bp, 2377 bp, and 229 bp following gel electrophoresis on a 1% agarose gel. L, 1 kb ladder. 1-20, the 20 pAGE1.0 vectors extracted from *E. coli* and digested. Notes: 1) the 229 bp band is very faint and barely visible in this image; 2) a total of 30 vectors were screened but only data for 20 is shown.

B.2 Supplemental Tables

Table B-1. List of strains used in this study.

Strain	Description	Resistance	Reference or Source
Sinorhizobium meliloti			
Rm5000	SU47 *rif-5*	Rif	[1]
RmP110	RmP1021, wild type SU47 *str-21* with wild-type *pstC* allele	Sm	[2]
RmP3491	Rm5000 (pTE3::*rctB* of *R. etli*)	Rif Tc	[3]
RmP3500	ΔpSymA ΔpSymB with *engA-tRNAarg-rmlC* moved to chromosome	Sm	[3]
RmP3901	RmP3500 with upstream and downstream regions of NGR234 *bacA* gene at *bacA* loci	Sm Gm	[4]
RmP3909	ΔpSymA ΔpSymB with *engA-tRNAarg-rmlC* moved to chromosome; *bacA* gene replaced with *bacA* from Rm2011; derived from RmP3500	Sm	[4]
RmP3910	RmP3901 with *bacA* gene replaced with NGR234 *bacA*	Sm	[4]
RmP3950	RmP3910 with pSymB from RmP3491	Sm	[4]
RmP3952	RmP3909 pSymA+ ΔpSymB	Sm	[4]
Rmp3953	RmP3909 ΔpSymA pSymB+	Sm	[4]
RmP3954	RmP3909 pSymA+ pSymB+	Sm	[4]
RmP3975	RmP110 (pTH3144) Δ*hsdR hsdR* gene replaced with Km/Nm cassette	Sm Nm	Zamani, unpublished.
RmP4098	RmP3950 ΔpSymA *hsdR*::Nm	Sm Nm	Zamani, unpublished.
RmP4122	RmP4098 ΔpSymA *hsdR*::FRT	Sm Nm^S	This study.
RmP4124	RmP3953 Δ*hsdR hsdR*::FRT	Sm Nm^S	This study.
RmP4125	RmP3954 Δ*hsdR hsdR*::FRT	Sm Nm^S	This study.
RmP4246	Rm5000 ΔpSymA	Rif	Bindra, Sather and Finan, unpublished.
RmP4258	RmP3909 Δ*hsdR hsdR*::FRT	Sm Nm^S	This study.
RmP4260	RmP3952 Δ*hsdR hsdR*::FRT	Sm Nm^S	This study.
RmP4262	RmP4246 Δ*hsdR hsdR*::FRT	Rif Nm^S	This study.
Escherichia coli			
DH5α	F- *endA1 hsdR17 supE44 thi-1 λ- recA1 gyrA96 relA1 Φ80dlacZΔM15*		[5]
MT616	MT607 (pRK600); contains RK2 transfer genes	Cm	[6]
M1449	DH5α (pTH2505)	Tc	[7]
βDH10B	Δ*dapA*		[8]
M2453	DH5α (pTH3142; *hsdR* upstream and downstream regions cloned in pUCP30T))	Gm	Zamani, unpublished
M2459	M2456 (Km cassette introduced to delete *hsdR*); pKD46	Gm Km Amp	Zamani, unpublished
ECGE101	Epi300 with the *dapA* gene deleted (Δ*dapA)*		This study.

Table B-2. List of oligonucleotides used in this study.

Name	Sequence (5' to 3')	Description
	Development of Δ*hsdR* strains	
Km_cas_F	CGACGTCTCGGTCGCCGAAGAGACCTTTGCCGAGG CTGCGGAATAGCCCGTGTAGGCTGGAGCTGCTTC	Forward Km cassette primer to delete *hsdR*
Km_cas_R	CTCAACCTGCCGTCTCCAGTGAAGGTGGCGGGGAG ACTGGAAGCCAAAGTATTCCGGGGATCCGTCGACC	Reverse Km cassette primer to delete *hsdR*
	Development of ECGE101	
DAP1	ATGTTCACGGGAAGTATTGTC	Forward *dapA* region from βDH10B
DAP2	CAGCAAACCGGCATGCTTAA	Reverse *dapA* region from βDH10B
	Diagnostic PCR of Δ*hsdR* strains	
hsdR_U_F	GGTACCCGGGGATCCTCTAGGACATAGCATCCGTA TCAGTTGGG	Forward primer for positive control in diagnostic colony PCR to validate deletion of *hsdR*
hsdR_U_R	GGTCAGGCTTCCGGCATTATCATCCCGCCGTCATCG TCG	Reverse primer for positive control in diagnostic colony PCR to validate deletion of *hsdR*
hsdM_U_F	GGTACCCGGGGATCCTCTAGCAAAGGACGGCACTT ATTCAACGG	Forward primer for diagnostic colony PCR to validate deletion of *hsdR*
hsdM_R_R	GGTCAGGCTTCCGGCATTATAGCCGGACGATGGAC GAGG	Reverse primer for diagnostic colony PCR to validate deletion of *hsdR*
hsdR_D_R	GCCTGCAGGTCGACTCTAGGATCAAGCTACTCGAT CAGGTGC	Reverse primer for diagnostic colony PCR to validate deletion of *hsdR*
	Assembly of MHS vectors	
D117_F	GTCCTTTTACAGCCAGTAGTGCTCGCCGCAGTCGAG CGACAGGGCGAAGCCCGTTTAAACCCGGGGATCCG GTGATTGAT	Forward Sp/Sm resistance cassette (pAGE1.0, pBGE1.0)
D117_R	TACCGAAAAAATCGCTATAATGACCCCGAAGCAGG GTTATGCAGCGGAAGATTTAATTAAGGATCCGGTG ATTGATTGAG	Reverse Sp/Sm resistance cassette (pAGE1.0, pBGE1.0)
D118_F	TTTCACAAAACGGTTTACAAGCATAAAGCTTGCTC AATCAATCACCGGATCCTTAATTAAATCTTCCGCTG CATAACCCT	Forward *oriT* (all pAGE and pBGE)
D118_R	CCTAAAAGACATAGCACGCGACAAGCACGAGCGA GATATCCCAATCAAGCTAGTATCGATGATCGTCTTG CCTTGCTCGT	Reverse *oriT* (all pAGE and pBGE)
D119_F	AGAAGAGGCACTTCGAGCTGTAAGTACATCACCGA CGAGCAAGGCAAGACGATCATCGATACTAGCTTGA TTGGGATATC	Forward Ntc resistance cassette (all pAGE and pBGE)
D119_R	GTCGCGATCGTGCGACGCCACGAACGGAGTGTTCG CTACCTTAGGACCGTTATAGTTACGGACGTTTTCAC TCTCGAGCA	Reverse Ntc resistance cassette (all pAGE and pBGE)
D120_F	TAAAAAACCTGTGCTCGAGAGTGAAAACGTCCGTA ACTATAACGGTCCTAAGGTAGCGAACACTCCGTTC GTGGCGTCGC	Forward *repA2B2C2* (all pAGE)
D120_R	CCAGCCCAGCGGCGAGGGCAACCAGCTCGACTATA	Reverse *repA2B2C2* (all

	TTACCCTGTTATCCCTAGCGTAACTGAACCATCCGG TGCAGTCGT	pAGE)
D121_F	TGACCGAGCAACGACTGCACCGGATGGTTCAGTTA CGCTAGGGATAACAGGGTAATATAGTCGAGCTGGT TGCCCTCGCC	Forward pCC1BAC-yeast part 1 (all pAGE and pBGE)
D121_R	TTCAACCGGTTGAGTATTGAGCGTATGTTTTGGAAT AACAGGCGCACGCTTCATTATCTAATCTCCCAGCGT GGTTTAAT	Reverse pCC1BAC-yeast part 1 (all pAGE and pBGE)
D122_F	ATTAAACCACGCTGGGAGATTAGATAATGAAGCGT GCGCCTGTTATTCCAAAACATACGCTCAATACTCAA CCGGTTGAA	Forward pCC1BAC-yeast part 2 (all pAGE and pBGE)
D122_R	ACAAAACGGTTTACAAGCATAAAGCTTGCTCAATC AATCACCGGATCCCCGGGTTTAAACGGGCTTCGCC CTGTCGCTCG	Reverse pCC1BAC-yeast part 2 (all pAGE and pBGE)
D200_F	AGCCAGTAGTGCTCGCCGCAGTCGAGCGACAGGGC GAAGCCCGTTTAAACGAGCGCCAGAAGGCCGCCAG AGAGGCCGAG	Forward Tet gene from pRK7813 (pAGE2.0, pBGE2.0)
D200_R	ATCGCTATAATGACCCCGAAGCAGGGTTATGCAGC GGAAGATTTAATTAAGATCAGACGCTGAGTGCGCT TCAAATCATC	Reverse Tet gene from pRK7813 (pAGE2.0, pBGE2.0)
D201_F	AGCCAGTAGTGCTCGCCGCAGTCGAGCGACAGGGC GAAGCCCGTTTAAACGGGGTGGGCGAAGAACTCCA GCATGAGATC	Forward NptII gene from pKNT253 (pAGE3.0, pBGE3.0)
D201_R	ATCGCTATAATGACCCCGAAGCAGGGTTATGCAGC GGAAGATTTAATTAAAGCTTCACGCTGCCGCAAGC ACTCAGGGCG	Reverse NptII gene from pKNT253 (pAGE3.0, pBGE3.0)
D234_F	GTGCTCGAGAGTGAAAACGTCCGTAACTATAACGG TCCTAAGGTAGCGAAGCCTTATCCTTCTCTTATCCG ACCTGCGGG	Forward *repA1B1C1* (all pBGE)
D234_R	GGCGAGGGCAACCAGCTCGACTATATTACCCTGTT ATCCCTAGCGTAACTGGGGCAAGATCGTATTGTTG ACAGCTGTCA	Reverse r*epA1B1C1* (all pBGE)

Table B-3. List of vectors used in this study.

Vector	Description	Resistance	Reference or Source
pUCP30T	ColE1 cloning vector in *S. meliloti, oriT*	Gm	[9]
pKD46	Expressed *lambda red recombinase* genes	Amp	[10]
pTH2505	Expresses *flp recombinase* under control of PCA-inducible promoter	Tc	[7]
pTA-Mob	Broad-host-range mobilization plasmid	Gm	[11]
pAGE1.0	MHS vector (as described in the main text)	Sp (*S. meliloti*) Cm (*E. coli*) HIS3 (*S. cerevisiae*) Ntc (*P. tricornutum)*	This study
pAGE2.0	MHS vector (as described in the main text)	Sp (*S. meliloti*) Cm (*E. coli*) HIS3 (*S. cerevisiae*) Ntc (*P. tricornutum)*	This study
pAGE3.0	MHS vector (as described in the main text)	Sp (*S. meliloti*) Cm (*E. coli*) HIS3 (*S. cerevisiae*) Ntc (*P. tricornutum)*	This study
pBGE1.0	MHS vector (as described in the main text)	Sp (*S. meliloti*) Cm (*E. coli*) HIS3 (*S. cerevisiae*) Ntc (*P. tricornutum)*	This study
pBGE2.0	MHS vector (as described in the main text)	Sp (*S. meliloti*) Cm (*E. coli*) HIS3 (*S. cerevisiae*) Ntc (*P. tricornutum)*	This study
pBGE3.0	MHS vector (as described in the main text)	Sp (*S. meliloti*) Cm (*E. coli*) HIS3 (*S. cerevisiae*) Ntc (*P. tricornutum)*	This study

References

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Appendix C: Supplemental Information for Chapter 3

C.1 Supplemental Figures

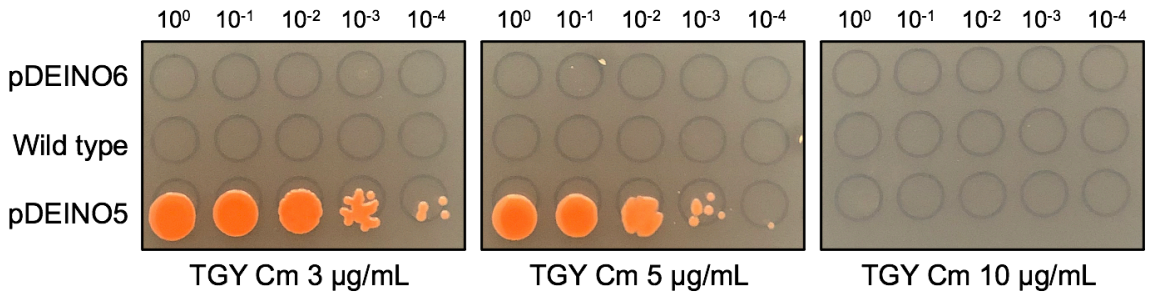

Figure C-1. *D. radiodurans* chloramphenicol sensitivity assay. Test of chloramphenicol resistance in *D. radiodurans* harbouring pDEINO6 (containing *E. coli cat* gene only) and pDEINO5 (containing *D. radiodurans* codon-optimized *cat* gene and *E. coli cat* gene) compared to wild type. A 10-fold dilution series of each strain was spot plated on TGY media supplemented with increasing concentration of chloramphenicol.

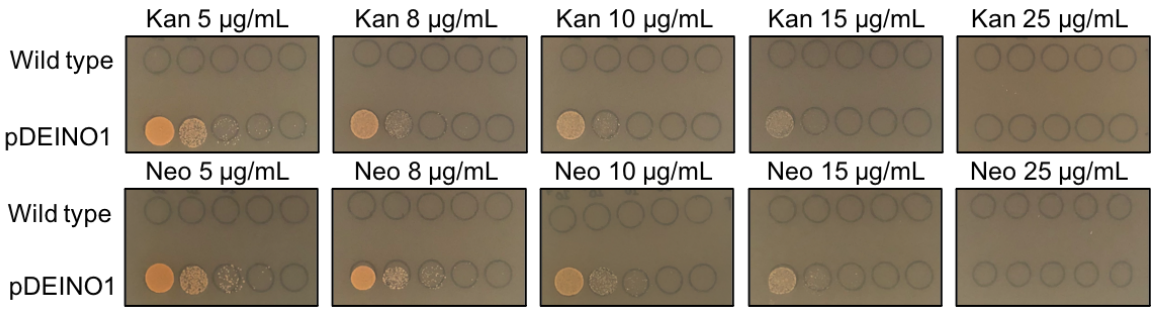

Figure C-2. *D. radiodurans* kanamycin and neomycin sensitivity assay. Test of kanamycin and neomycin sensitivity of *D. radiodurans* harbouring pDEINO1 compared to wild type. A 10-fold dilution series of each strain was spot plated on TGY media supplemented with increasing concentration of antibiotic.

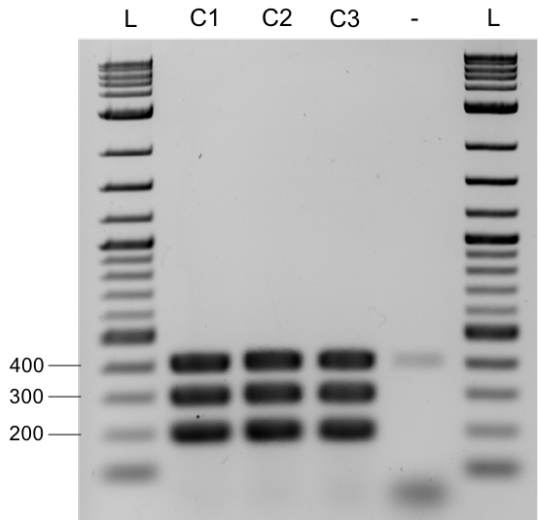

Figure C-3. PCR analysis of pDEINO2 integration in *D. radiodurans* transconjugants. Agarose gel of multiplex PCR performed on DNA extracted from three *D. radiodurans* transconjugant colonies following conjugation of pDEINO2 to clone the MP1 megaplasmid. Amplicons from the integrated pDEINO2 plasmid should be 200, 300 and 400 bp in size. L, 2-log ladder. -, water.

C.2 Supplemental Tables

Table C-1. List of plasmids used in this study.

Plasmid	Description	Resistance	Reference or Source
pAGE1.0	MHS vector	Cm (*E. coli*), HIS3 (*S. cerevisiae*), Str/Spec (*S. meliloti*), Ntc (*P. tricornutum*)	Brumwell et al., 2019
pAGE2.0	MHS vector	Cm (*E. coli*), HIS3 (*S. cerevisiae*), Tet (*S. meliloti*), Ntc (*P. tricornutum*)	Brumwell et al., 2019
pAGE3.0	MHS vector	Cm (*E. coli*), HIS3 (*S. cerevisiae*), Nm (*S. meliloti*), Ntc (*P. tricornutum*)	Brumwell et al., 2019
pRAD1	General cloning vector for use in *E. coli* or *D. radiodurans*	Amp (*E. coli),* Cm (*D. radiodurans*)	Meima et al., 2000
pTA-Mob	Broad-host-range mobilization plasmid	Gm (*E. coli)*	Strand et al., 2014
pTA-Mob 2.0 Δ*traI*	pTA-Mob derivative with an *oriT* and *traI* deletion	Gm (*E. coli*)	Unpublished, Karas Lab
pRadDEST-GFP	Replicating plasmid with GFP	Cm (*D. radiodurans*)	Unpublished, Junop Lab
pDEINO1	pAGE3.0 with *D. radiodurans* origin and codon-optimized Cm marker	Cm (*D. radiodurans*), Cm (*E. coli*), HIS3 (*S. cerevisiae*), Nm (*S. meliloti* and *D. radiodurans*), Ntc (*P. tricornutum*)	This study Addgene ID: 179472
pDEINO2	Nonreplicating plasmid with 1 kb homology to *McrC*	Cm (*D. radiodurans*), Cm (*E. coli*), HIS3 (*S. cerevisiae*)	This study
pDEINO3	Replicating plasmid with Tet selection marker	Tet and Cm (*D. radiodurans*), Cm (*E. coli*), HIS3 (*S. cerevisiae*)	This study Addgene ID: 179487
pDEINO4	Replicating plasmid with Str selection marker	Str and Cm (*D. radiodurans*), Cm (*E. coli*), HIS3 (*S. cerevisiae*)	This study Addgene ID: 179488
pDEINO5	Replicating plasmid with Gm selection marker	Gm and Cm (*D. radiodurans*), Cm (*E. coli*), HIS3 (*S. cerevisiae*)	This study Addgene ID: 179489)
pDEINO6	Replicating plasmid with Tet selection marker and without *D. radiodurans* Cm	Tet (*D. radiodurans*), Cm (*E. coli*), HIS3 (*S. cerevisiae*)	This study
pDEINO7	Nonreplicating plasmid with two 1 kb homology regions flanking ORF14075	Nm (*D. radiodurans*), Cm (*E. coli*), HIS3 (*S. cerevisiae*)	This study
pDEINO8	Nonreplicating plasmid with two 1 kb homology regions flanking ORF15360	Tet (*D. radiodurans*), Cm (*E. coli*), HIS3 (*S. cerevisiae*)	This study
pDEINO9	Nonreplicating plasmid with two 1 kb homology regions flanking *Mrr*	Tet (*D. radiodurans*), Cm (*E. coli*), HIS3 (*S. cerevisiae*)	This study
pDEINO10	Nonreplicating plasmid with two 1 kb homology regions flanking ORF2230	Nm (*D. radiodurans*), Cm (*E. coli*), HIS3 (*S. cerevisiae*)	This study

Table C-2. List of oligonucleotides used in this study. The bold, underlined sequence in the assembly primers represents the binding portion of the primer, while the remainder of the sequence is the hook or homology region to the adjacent fragment.

Name	Sequence (5' to 3')	Description
	pDEINO1 Assembly Primers	
BK486_F	GTTCCGCTTCCTTTAGCAGCCCTTGCGCCCTGA GTGCTTGCGGCAGCGTGAAGCTTTAAT**CATGA TTACGCCAAGCTCGC**	pDEINO1 assembly primer (origin from pRAD1)
BK486_R	ATATTGAGAATCATTCTCAATGTCCAGGGCCC TCGGTCTCCATGGCCCTCAGGCCCTCGC**TTTA GCTTCCTTAGCTCCTG**	pDEINO1 assembly primer (origin from pRAD1)
BK487_F	GCTTGAGTAGGACAAATCCGCCGAGCTTCGAC GAGATTTTCAGGAGCTAAGGAAGCTAAA**GCG AGGGCCTGAGGGCCATG**	pDEINO1 assembly primer ($DrCm^R$)
BK487_R	GGATATACCGAAAAAATCGCTATAATGACCCC GAAGCAGGGTTATGCAGCGGAAGATTTA**AAA AAACCCCCCGGATTGCC**	pDEINO1 assembly primer ($DrCm^R$)
	pDEINO2 Assembly Primers	
BK1388_F	AGTACATCACCGACGAGCAAGGCAAGACGAT CTTAATTAA**TCGAGCTGGTTGCCCTCGCC**	pDEINO2 assembly primer (split pCC1BAC-yeast #1)
BK1388_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAAC AGTTCTCA**TCCGGATCTGACCTTTACCA**	pDEINO2 assembly primer (split pCC1BAC-yeast #1)
BK1389_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCA GATCCGGA**TGAGAACTGTTTTTGAACAG**	pDEINO2 assembly primer (split pCC1BAC-yeast #2)
BK1409_R	CAAATGCTCGCCTATAGCGAGGCCTTTCAGCA CAGCGCGG**GTTTAAACGGGCTTCGCCCT**	pDEINO2 assembly primer (split pCC1BAC-yeast #2)
BK1410_F	GCTCGCCGCAGTCGAGCGACAGGGCGAAGCC CGTTTAAAC**CCGCGCTGTGCTGAAAGGCC**	pDEINO2 assembly primer (1 kb homology to DraR1McrCP)
BK1410_R	GCCCTCGGTCTCCATGGCCCTCAGGCCCTCGC GGCGCGCC**CTGAGCCCGGTGGCCACCAT**	pDEINO2 assembly primer (1 kb homology to DraR1McrCP)
BK1411_F	TCAAAGCGGGTCATGGTGGCCACCGGGCTCAG GGCGCGCC**GCGAGGGCCTGAGGGCCATG**	pDEINO2 assembly primer ($DrCm^R$ and *oriT*)
BK1392_R	CGCCAGCCCAGCGGCGAGGGCAACCAGCTCG ATTAATTAA**GATCGTCTTGCCTTGCTCGT**	pDEINO2 assembly primer ($DrCm^R$ and *oriT*)
	pDEINO3 Assembly Primers	
BK1388_F	AGTACATCACCGACGAGCAAGGCAAGACGAT CTTAATTAA**TCGAGCTGGTTGCCCTCGCC**	pDEINO3 assembly primer (split pCC1BAC-yeast #1)
BK1388_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAAC AGTTCTCA**TCCGGATCTGACCTTTACCA**	pDEINO3 assembly primer (split pCC1BAC-yeast #1)
BK1389_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCA GATCCGGA**TGAGAACTGTTTTTGAACAG**	pDEINO3 assembly primer (split pCC1BAC-yeast #2)
BK1389_R	TCGATAGATCTCGAGGCCTCGCGAGCTTGGCG TAATCATG**GTTTAAACGGGCTTCGCCCT**	pDEINO3 assembly primer (split pCC1BAC-yeast #2)
BK1390_F	GCTCGCCGCAGTCGAGCGACAGGGCGAAGCC CGTTTAAAC**CATGATTACGCCAAGCTCGC**	pDEINO3 assembly primer (Drad origin)
BK1694_R	CACGGCCGCGCTCGGCCTCTCTGGCGGCCTTC TGGCGCTC**TTTAGCTTCCTTAGCTCCTG**	pDEINO3 assembly primer (Drad origin)
BK1695_F	CCGAGCTTCGACGAGATTTTCAGGAGCTAAGG AAGCTAAA**GAGCGCCAGAAGGCCGCCAG**	pDEINO3 assembly primer (Tet^R)

BK1695_R	TGTCCAGGGCCCTCGGTCTCCATGGCCCTCAG GCCCTCGC**GATCAGACGCTGAGTGCGCT**	pDEINO3 assembly primer (Tet^R)
BK1696_F	GCAGGACGCCGATGATTTGAAGCGCACTCAGC GTCTGATC**GCGAGGGCCTGAGGGCCATG**	pDEINO3 assembly primer ($DrCm^R$ and *oriT*)
BK1392_R	CGCCAGCCCAGCGGCGAGGGCAACCAGCTCG ATTAATTAA**GATCGTCTTGCCTTGCTCGT**	pDEINO3 assembly primer ($DrCm^R$ and *oriT*)
	pDEINO4 Assembly Primers	
BK1388_F	AGTACATCACCGACGAGCAAGGCAAGACGAT CTTAATTAA**TCGAGCTGGTTGCCCTCGCC**	pDEINO4 assembly primer (split pCC1BAC-yeast #1)
BK1388_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAAC AGTTCTCA**TCCGGATCTGACCTTTACCA**	pDEINO4 assembly primer (split pCC1BAC-yeast #1)
BK1389_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCA GATCCGGA**TGAGAACTGTTTTTGAACAG**	pDEINO4 assembly primer (split pCC1BAC-yeast #2)
BK1389_R	TCGATAGATCTCGAGGCCTCGCGAGCTTGGCG TAATCATG**GTTTAAACGGGCTTCGCCCT**	pDEINO4 assembly primer (split pCC1BAC-yeast #2)
BK1390_F	GCTCGCCGCAGTCGAGCGACAGGGCGAAGCC CGTTTAAAC**CATGATTACGCCAAGCTCGC**	pDEINO4 assembly primer (Drad origin)
BK1697_R	ACAAGCATAAAGCTTGCTCAATCAATCACCGG ATCCCCGG**TTTAGCTTCCTTAGCTCCTG**	pDEINO4 assembly primer (Drad origin)
BK1698_F	CCGAGCTTCGACGAGATTTTCAGGAGCTAAGG AAGCTAAA**CCGGGGATCCGGTGATTGAT**	pDEINO4 assembly primer (Str^R)
BK1698_R	TGTCCAGGGCCCTCGGTCTCCATGGCCCTCAG GCCCTCGC**GGATCCGGTGATTGATTGAG**	pDEINO4 assembly primer (Str^R)
BK1699_F	GTTTACAAGCATAAAGCTTGCTCAATCAATCA CCGGATCC**GCGAGGGCCTGAGGGCCATG**	pDEINO4 assembly primer ($DrCm^R$ and *oriT*)
BK1392_R	CGCCAGCCCAGCGGCGAGGGCAACCAGCTCG ATTAATTAA**GATCGTCTTGCCTTGCTCGT**	pDEINO4 assembly primer ($DrCm^R$ and *oriT*)
	pDEINO5 Assembly Primers	
BK1388_F	AGTACATCACCGACGAGCAAGGCAAGACGAT CTTAATTAA**TCGAGCTGGTTGCCCTCGCC**	pDEINO5 assembly primer (split pCC1BAC-yeast #1)
BK1388_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAAC AGTTCTCA**TCCGGATCTGACCTTTACCA**	pDEINO5 assembly primer (split pCC1BAC-yeast #1)
BK1389_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCA GATCCGGA**TGAGAACTGTTTTTGAACAG**	pDEINO5 assembly primer (split pCC1BAC-yeast #2)
BK1389_R	TCGATAGATCTCGAGGCCTCGCGAGCTTGGCG TAATCATG**GTTTAAACGGGCTTCGCCCT**	pDEINO5 assembly primer (split pCC1BAC-yeast #2)
BK1390_F	GCTCGCCGCAGTCGAGCGACAGGGCGAAGCC CGTTTAAAC**CATGATTACGCCAAGCTCGC**	pDEINO5 assembly primer (Drad origin)
BK2117_R	CCCGGCCGCGGAGTTGTTCGGTAAATTGTCAC AACGCCGC**TTTAGCTTCCTTAGCTCCTG**	pDEINO5 assembly primer (Drad origin)
BK2118_F	CCGAGCTTCGACGAGATTTTCAGGAGCTAAGG AAGCTAAA**GCGGCGTTGTGACAATTTAC**	pDEINO5 assembly primer (Gm^R)
BK2118_R	TGTCCAGGGCCCTCGGTCTCCATGGCCCTCAG GCCCTCGC**GACGCACACCGTGGAAACGG**	pDEINO5 assembly primer (Gm^R)
BK2119_F	AACTGGGTTCGTGCCTTCATCCGTTTCCACGGT GTGCGTC**GCGAGGGCCTGAGGGCCATG**	pDEINO5 assembly primer ($DrCm^R$ and *oriT*)
BK1392_R	CGCCAGCCCAGCGGCGAGGGCAACCAGCTCG ATTAATTAA**GATCGTCTTGCCTTGCTCGT**	pDEINO5 assembly primer ($DrCm^R$ and *oriT*)
	pDEINO6 Assembly Primers	
BK1388_F	AGTACATCACCGACGAGCAAGGCAAGACGAT CTTAATTAA**TCGAGCTGGTTGCCCTCGCC**	pDEINO6 assembly primer (split pCC1BAC-yeast #1)

BK1388_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAAC AGTTCTCA**TCCGGATCTGACCTTTACCA**	pDEINO6 assembly primer (split pCC1BAC-yeast #1)
BK1389_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCA GATCCGGA**TGAGAACTGTTTTTGAACAG**	pDEINO6 assembly primer (split pCC1BAC-yeast #2)
BK1389_R	TCGATAGATCTCGAGGCCTCGCGAGCTTGGCG TAATCATG**GTTTAAACGGGCTTCGCCCT**	pDEINO6 assembly primer (split pCC1BAC-yeast #2)
BK1390_F	GCTCGCCGCAGTCGAGCGACAGGGCGAAGCC CGTTTAAAC**CATGATTACGCCAAGCTCGC**	pDEINO6 assembly primer (Drad origin)
BK1694_R	CACGGCCGCGCTCGGCCTCTCTGGCGGCCTTC TGGCGCTC**TTTAGCTTCCTTAGCTCCTG**	pDEINO6 assembly primer (Drad origin)
BK1695_F	CCGAGCTTCGACGAGATTTTCAGGAGCTAAGG AAGCTAAA**GAGCGCCAGAAGGCCGCCAG**	pDEINO6 assembly primer (Tet^R)
BK2103_R	CGCTATAATGACCCCGAAGCAGGGTTATGCAG CGGAAGAT**GATCAGACGCTGAGTGCGCT**	pDEINO6 assembly primer (Tet^R)
BK2104_F	GCAGGACGCCGATGATTTGAAGCGCACTCAGC GTCTGATC**ATCTTCCGCTGCATAACCCT**	pDEINO6 assembly primer (*oriT*)
BK1392_R	CGCCAGCCCAGCGGCGAGGGCAACCAGCTCG ATTAATTAA**GATCGTCTTGCCTTGCTCGT**	pDEINO6 assembly primer (*oriT*)
	pDEINO7 Assembly Primers	
BK1388_F	AGTACATCACCGACGAGCAAGGCAAGACGAT CTTAATTAA**TCGAGCTGGTTGCCCTCGCC**	pDEINO7 assembly primer (split pCC1BAC-yeast #1)
BK1388_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAAC AGTTCTCA**TCCGGATCTGACCTTTACCA**	pDEINO7 assembly primer (split pCC1BAC-yeast #1)
BK1389_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCA GATCCGGA**TGAGAACTGTTTTTGAACAG**	pDEINO7 assembly primer (split pCC1BAC-yeast #2)
BK1869_R	GTACGAGCAGGAACTGGGATTCATTACCCTGT TATCCCTA**GGGCTTCGCCCTGTCGCTCG**	pDEINO7 assembly primer (split pCC1BAC-yeast #2)
BK1870_F	GTCGAGCGACAGGGCGAAGCCCTAGGGATAA CAGGGTAAT**GAATCCCAGTTCCTGCTCGT**	pDEINO7 assembly primer (Mrr homology #1)
BK2124_R	CACGGCCGCGCTCGGCCTCTCTGGCGGCCTTC TGGCGCTC**ATTTTTTAAGTTTACGCTCT**	pDEINO7 assembly primer (Mrr homology #1)
BK2125_F	CGAAAGCCGTAAGTCCAGACAGAGCGTAAAC TTAAAAAAT**GAGCGCCAGAAGGCCGCCAG**	pDEINO7 assembly primer (Tet^R)
BK2125_R	CCTCCTCCCTTGACGCCGGAAACCGCCCTGTC AGGCGAGC**GATCAGACGCTGAGTGCGCT**	pDEINO7 assembly primer (Tet^R)
BK2126_F	GCAGGACGCCGATGATTTGAAGCGCACTCAGC GTCTGATC**GCTCGCCTGACAGGGCGGTT**	pDEINO7 assembly primer (Mrr homology #2)
BK1872_R	GCAGGGTTATGCAGCGGAAGATATTACCCTGT TATCCCTA**CGGCAGTTCCACGTTGCACA**	pDEINO7 assembly primer (Mrr homology #2)
BK1873_F	GTTGTGCAACGTGGAACTGCCGTAGGGATAAC AGGGTAAT**ATCTTCCGCTGCATAACCCT**	pDEINO7 assembly primer (*oriT*)
BK1392_R	CGCCAGCCCAGCGGCGAGGGCAACCAGCTCG ATTAATTAA**GATCGTCTTGCCTTGCTCGT**	pDEINO7 assembly primer (*oriT*)
	pDEINO8 Assembly Primers	
BK1388_F	AGTACATCACCGACGAGCAAGGCAAGACGAT CTTAATTAA**TCGAGCTGGTTGCCCTCGCC**	pDEINO8 assembly primer (split pCC1BAC-yeast #1)
BK1388_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAAC AGTTCTCA**TCCGGATCTGACCTTTACCA**	pDEINO8 assembly primer (split pCC1BAC-yeast #1)
BK1389_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCA GATCCGGA**TGAGAACTGTTTTTGAACAG**	pDEINO8 assembly primer (split pCC1BAC-yeast #2)

BK1864_R	GCGAGCATGTACGCCTGGGCGCATTACCCTGT TATCCCTA**GGGCTTCGCCCTGTCGCTCG**	pDEINO8 assembly primer (split pCC1BAC-yeast #2)
BK1865_F	GTCGAGCGACAGGGCGAAGCCCTAGGGATAA CAGGGTAAT**GCGCCCAGGCGTACATGCTC**	pDEINO8 assembly primer (ORF2230 homology #1)
BK2127_R	CCAGCGCGGGGATCTCATGCTGGAGTTCTTCG CCCACCCC**GAACCTCTTCAGAGTACGGC**	pDEINO8 assembly primer (ORF2230 homology #1)
BK2128_F	CCCACATGGCCCGAGTGTAAGCCGTACTCTGA AGAGGTTC**GGGGTGGGCGAAGAACTCCA**	pDEINO8 assembly primer ($NptII^R$)
BK2128_R	CGGGCACCCGGGAGCTTCCCTGGGTGCCCGCT TTCGGTGT**AGCTTCACGCTGCCGCAAGC**	pDEINO8 assembly primer ($NptII^R$)
BK2129_F	GCAGCCCTTGCGCCCTGAGTGCTTGCGGCAGC GTGAAGCT**ACACCGAAAGCGGGCACCCA**	pDEINO8 assembly primer (ORF2230 homology #2)
BK1867_R	GCAGGGTTATGCAGCGGAAGATATTACCCTGT TATCCCTA**CGTGAGCACCATTTTCATCC**	pDEINO8 assembly primer (ORF2230 homology #2)
BK1868_F	CAGGATGAAAATGGTGCTCACGTAGGGATAA CAGGGTAAT**ATCTTCCGCTGCATAACCCT**	pDEINO8 assembly primer (*oriT*)
BK1392_R	CGCCAGCCCAGCGGCGAGGGCAACCAGCTCG ATTAATTAA**GATCGTCTTGCCTTGCTCGT**	pDEINO8 assembly primer (*oriT*)
	pDEINO9 Assembly Primers	
BK1388_F	AGTACATCACCGACGAGCAAGGCAAGACGAT CTTAATTAA**TCGAGCTGGTTGCCCTCGCC**	pDEINO9 assembly primer (split pCC1BAC-yeast #1)
BK1388_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAAC AGTTCTCA**TCCGGATCTGACCTTTACCA**	pDEINO9 assembly primer (split pCC1BAC-yeast #1)
BK1389_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCA GATCCGGA**TGAGAACTGTTTTTGAACAG**	pDEINO9 assembly primer (split pCC1BAC-yeast #2)
BK1874_R	ACCTTGCGGTACCGGGCGAGGCATTACCCTGT TATCCCTA**GGGCTTCGCCCTGTCGCTCG**	pDEINO9 assembly primer (split pCC1BAC-yeast #2)
BK1875_F	GTCGAGCGACAGGGCGAAGCCCTAGGGATAA CAGGGTAAT**GCCTCGCCCGGTACCGCAAG**	pDEINO9 assembly primer (ORF14075 homology #1)
BK1875_R	CCAGCGCGGGGATCTCATGCTGGAGTTCTTCG CCCACCCC**ATCAGCTCGCAGGCTAGCGC**	pDEINO9 assembly primer (ORF14075 homology #1)
BK1876_F	CTCCGACTGACTTTGCTGCTGCGCTAGCCTGC GAGCTGAT**GGGGTGGGCGAAGAACTCCA**	pDEINO9 assembly primer ($NptII^R$)
BK1876_R	CAAGAAAGCCTCGTCGTAAGGCGAGTACGAA TCTCAAGCT**AGCTTCACGCTGCCGCAAGC**	pDEINO9 assembly primer ($NptII^R$)
BK1877_F	GCAGCCCTTGCGCCCTGAGTGCTTGCGGCAGC GTGAAGCT**AGCTTGAGATTCGTACTCGC**	pDEINO9 assembly primer (ORF14075 homology #2)
BK1877_R	GCAGGGTTATGCAGCGGAAGATATTACCCTGT TATCCCTA**CCCAGACCTGCTCCGGCGTG**	pDEINO9 assembly primer (ORF14075 homology #2)
BK1878_F	GGCACGCCGGAGCAGGTCTGGGTAGGGATAA CAGGGTAAT**ATCTTCCGCTGCATAACCCT**	pDEINO9 assembly primer (*oriT*)
BK1392_R	CGCCAGCCCAGCGGCGAGGGCAACCAGCTCG ATTAATTAA**GATCGTCTTGCCTTGCTCGT**	pDEINO9 assembly primer (*oriT*)
	pDEINO10 Assembly Primers	
BK1388_F	AGTACATCACCGACGAGCAAGGCAAGACGAT CTTAATTAA**TCGAGCTGGTTGCCCTCGCC**	pDEINO10 assembly primer (split pCC1BAC-yeast #1)
BK1388_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAAC AGTTCTCA**TCCGGATCTGACCTTTACCA**	pDEINO10 assembly primer (split pCC1BAC-yeast #1)
BK1389_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCA GATCCGGA**TGAGAACTGTTTTTGAACAG**	pDEINO10 assembly primer (split pCC1BAC-yeast #2)

BK1879_R	CAGTCCTCGGAAACTTCTGCCCATTACCCTGTT ATCCCTA**GGGCTTCGCCCTGTCGCTCG**	pDEINO10 assembly primer (split pCC1BAC-yeast #2)
BK1880_F	GTCGAGCGACAGGGCGAAGCCCTAGGGATAA CAGGGTAAT**GGGCAGAAGTTTCCGAGGAC**	pDEINO10 assembly primer (ORF15360 homology #1)
BK1880_R	CACGGCCGCGCTCGGCCTCTCTGGCGGCCTTC TGGCGCTC**ACAATGCCATTTATGTTTTC**	pDEINO10 assembly primer (ORF15360 homology #1)
BK1881_F	CAGCTAGTGCCGAAGTGCCAGAAAACATAAA TGGCATTGT**GAGCGCCAGAAGGCCGCCAG**	pDEINO10 assembly primer (Tet^R)
BK1881_R	AACTTTCCGACCGCGCCCAGACGGGCAAGGTC GAGGGGGC**GATCAGACGCTGAGTGCGCT**	pDEINO10 assembly primer (Tet^R)
BK1882_F	GCAGGACGCCGATGATTTGAAGCGCACTCAGC GTCTGATC**GCCCCCTCGACCTTGCCCGT**	pDEINO10 assembly primer (ORF15360 homology #2)
BK1882_R	GCAGGGTTATGCAGCGGAAGATATTACCCTGT TATCCCTA**GTGATTCAGCCGCTGCTCTT**	pDEINO10 assembly primer (ORF15360 homology #2)
BK1883_F	CAAAGAGCAGCGGCTGAATCACTAGGGATAA CAGGGTAAT**ATCTTCCGCTGCATAACCCT**	pDEINO10 assembly primer (*oriT*)
BK1392_R	CGCCAGCCCAGCGGCGAGGGCAACCAGCTCG ATTAATTAA**GATCGTCTTGCCTTGCTCGT**	pDEINO10 assembly primer (*oriT*)
	pDEINO1 MPX Primers	
BK1327_F	GTGGAGCGGATTATGTCAGCAATG	pDEINO1 MPX primers – 200 bp (junction between pCC1BAC-yeast and NptII)
BK1327_R	CGGAATCGTTTTCCGGGACG	pDEINO1 MPX primers – 200 bp (junction between pCC1BAC-yeast and NptII)
BK1328_F	CCGCAATACCGGCTTCGCT	pDEINO1 MPX primers – 300 bp (within RepA2B2C2)
BK1328_R	CTCGAGGGCCTCTTCTGGAAGG	pDEINO1 MPX primers – 300 bp (within RepA2B2C2)
BK1329_F	GCAGTAGCAGAACAGGCCACAC	pDEINO1 MPX primers – 400 bp (within pCC1BAC-yeast)
BK1329_R	GGGTAATTCTGCTAGCCTCTGCAA	pDEINO1 MPX primers – 400 bp (within pCC1BAC-yeast)
BK1330_F	GCCATCCCCCTGCTCTACGA	pDEINO1 MPX primers – 650 bp (junction between ori and KatA promoter)
BK1330_R	GTGATACTCCGACCAGAGAGACGA	pDEINO1 MPX primers – 650 bp (junction between ori and KatA promoter)
	pDEINO2 MPX Primers	
BK1327_F	GTGGAGCGGATTATGTCAGCAATG	pDEINO2 MPX primers – 200 bp (pCC1BAC-yeast and 1 kb homology junction)
BK1428_R	GCCCCCACCTACGACGTG	pDEINO2 MPX primers – 200 bp (pCC1BAC-yeast and 1 kb homology junction)
BK1412_F	GTGGACATTAGTCAGTGGCATCGT	pDEINO2 MPX primers – 300 bp (within $DrCm^R$)
BK1330_R	GTGATACTCCGACCAGAGAGACGA	pDEINO2 MPX primers – 300 bp (within $DrCm^R$)
BK1329_F	GCAGTAGCAGAACAGGCCACAC	pDEINO2 MPX primers – 400 bp (within pCC1BAC-yeast)

BK1329_R	GGGTAATTCTGCTAGCCTCTGCAA	pDEINO2 MPX primers – 400 bp (within pCC1BAC-yeast)
MP1 Megaplasmid MPX Primers		
BK1520_F	GGCAGCTTCAGGGACGTGTC	MP1 MPX Primers– 200 bp
BK1520_R	CTCCATGTCTTTCCGCTTGGAGG	MP1 MPX Primers – 200 bp
BK1521_F	GTATGGGCCCTGACGGCC	MP1 MPX Primers – 300 bp
BK1521_R	GCTGGCCGAACTGGAAGAGG	MP1 MPX Primers – 300 bp
BK1522_F	GATCCACCAGGCGAGGGC	MP1 MPX Primers – 400 bp
BK1522_R	CTATATCGAGGGCAGCGGCC	MP1 MPX Primers – 400 bp
BK1523_F	GATGGACCTGGGAAGCGCC	MP1 MPX Primers – 600 bp
BK1523_R	CGGGTTGTGCGTCAATTCGC	MP1 MPX Primers – 600 bp
pDEINO7 MPX Primers		
BK2001_F	CTGATTGTCATCAGCGCATT	Tet^R
BK2001_R	CAAAGTTGCAGCCGAATACA	Tet^R
BK2005_F	ACGACCATCACACCACTGAA	pCC1BAC-yeast backbone
BK2005_R	CATGACCAGCGTTTATGCAC	pCC1BAC-yeast backbone
BK2216_F	CCTGACCGAAAGAGAGTTCG	Mrr
BK2216_R	GTAGCGGGAGGTCGTCATAA	Mrr
pDEINO8 MPX Primers		
BK2000_F	CGAAACGATCCTCATCCTGT	Nm^R
BK2000_R	AGGAAGCGGAACACGTAGAA	Nm^R
BK2002_F	GAAGAACTGCCTGAGCGGTA	ORF2230
BK2002_R	GTCCATGCTGCTCTGAAACA	ORF2230
BK2005_F	ACGACCATCACACCACTGAA	pCC1BAC-yeast backbone
BK2005_R	CATGACCAGCGTTTATGCAC	pCC1BAC-yeast backbone
pDEINO9 MPX Primers		
BK2000_F	CGAAACGATCCTCATCCTGT	Nm^R
BK2000_R	AGGAAGCGGAACACGTAGAA	Nm^R

BK2003_F	GGCCCACTTCATCACAGAGT	ORF14075
BK2003_R	CCGAACAGGTCCTGGAAGTA	ORF14075
BK2005_F	ACGACCATCACACCACTGAA	pCC1BAC-yeast backbone
BK2005_R	CATGACCAGCGTTTATGCAC	pCC1BAC-yeast backbone
pDEINO10 MPX Primers		
BK2001_F	CTGATTGTCATCAGCGCATT	Tet^R
BK2001_R	CAAAGTTGCAGCCGAATACA	Tet^R
BK2004_F	GCTGGTAAATGCCCTTCGTA	ORF15360
BK2004_R	TCTACGCCGACTTCCTGTTC	ORF15360
BK2005_F	ACGACCATCACACCACTGAA	pCC1BAC-yeast backbone
BK2005_R	CATGACCAGCGTTTATGCAC	pCC1BAC-yeast backbone

Appendix D: Supplemental Information for Chapter 4

D.1 Supplemental Tables

Table D-1. *Deinococcus radiodurans* strains created in this study.

Strain	Description	Resistance	Reference or Source
ΔRM1	ΔORF14075	None	This study
ΔRM1-2	ΔORF14075 Δ*Mrr*	None	This study
ΔRM1-3	ΔORF14075 Δ*Mrr* ΔORF15360	None	This study
ΔRM1-4	ΔORF14075 Δ*Mrr* ΔORF15360 Δ*Mrr2*	None	This study
ΔRM1-5 Nm	ΔORF14075 Δ*Mrr* ΔORF15360 Δ*Mrr2* ΔORF2230	Nm	This study

Table D-2. List of plasmids used in this study.

Plasmid	Description	Resistance	Reference or Source
pBH474	Suc^s derivative of pTH474		(1)
pDEINO1	Replicating plasmid with codon-optimized Cm marker	Cm (*D. radiodurans, E. coli*) Nm (*D. radiodurans, E. coli, S. meliloti*) HIS3 (*S. cerevisiae*) Ntc (*P. tricornutum*)	(2) Addgene ID: 179472
pDEINO3	Replicating plasmid with Tet marker	Tet and Cm (*D. radiodurans, E. coli*) HIS3 (*S. cerevisiae*)	(2) Addgene ID: 179487
pDEINO4	Replicating plasmid with Strep marker	Cm (*D. radiodurans, E. coli*) Strep (*D. radiodurans*) HIS3 (*S. cerevisiae*)	(2) Addgene ID: 179488
pDEINO10	Nonreplicating plasmid with two 1 kb homology regions flanking ORF2230	Nm (*D. radiodurans*), Cm (*E. coli*), HIS3 (*S. cerevisiae*)	(2)
pSLICER	Replicating SLICER plasmid containing *I-SceI* endonuclease	Cm (*D. radiodurans, E. coli*) HIS3 (*S. cerevisiae*)	This study
pSD1	Non-replicating plasmid containing RM1 SD cassette	Nm and Tet (*D. radiodurans, E. coli*) Cm (*E. coli*) HIS3 (*S. cerevisiae*)	This study
pSD2	Non-replicating plasmid containing RM2 SD cassette	Nm (*D. radiodurans, E. coli*) Strep (*D. radiodurans*) Cm (*E. coli*) HIS3 (*S. cerevisiae*)	This study
pSD3	Non-replicating plasmid containing RM3 SD cassette	Nm and Tet (*D. radiodurans, E. coli*) HIS3 (*S. cerevisiae*)	This study
pSD4	Non-replicating plasmid containing RM4 SD cassette	Nm and Tet (*D. radiodurans, E. coli*) HIS3 (*S. cerevisiae*)	This study
pET-24α(+)-lacZ	pET-24α(+) with *lacZ* under a constitutive promoter	Kan (*E. coli*) HIS3 (*S. cerevisiae*)	Pellegrino, unpublished
pRAD1	General cloning vector for use in *E. coli* or *D. radiodurans*	Amp (*E. coli),* Cm (*D. radiodurans*)	(3)
pTA-Mob	Broad-host-range mobilization plasmid	Gm (*E. coli)*	(4)

Table D-3. List of oligonucleotides used in this study. The bold, underlined sequence in the assembly primers represents the binding portion of the primer, while the remainder of the sequence is the hook (*i.e.,* homology region) to the adjacent fragment. Sequence in red indicates the *I-Sce*I recognition site.

Name	Sequence (5' to 3')	Description
pSLICER Assembly Primers		
BK1388_F	AGTACATCACCGACGAGCAAGGCAAGACGATC TTAATTAA**TCGAGCTGGTTGCCCTCGCC**	pSLICER assembly primer (split pCC1BAC-yeast #1)
BK1388_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAAC AGTTCTCA**TCCGGATCTGACCTTTACCA**	pSLICER assembly primer (split pCC1BAC-yeast #1)
BK1389_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCA GATCCGGA**TGAGAACTGTTTTTGAACAG**	pSLICER assembly primer (split pCC1BAC-yeast #2)
BK1389_R	TCGATAGATCTCGAGGCCTCGCGAGCTTGGCG TAATCATG**GTTTAAACGGGCTTCGCCCT**	pSLICER assembly primer (split pCC1BAC-yeast #2)
BK1390_F	GCTCGCCGCAGTCGAGCGACAGGGCGAAGCCC GTTTAAAC**CATGATTACGCCAAGCTCGC**	pSLICER assembly primer (Drad origin)
BK1945_R	TGTCCAGGGCCCTCGGTCTCCATGGCCCTCAG GCCCTCGC**TTTAGCTTCCTTAGCTCCTG**	pSLICER assembly primer (Drad origin)
BK1946_F	CCGAGCTTCGACGAGATTTTCAGGAGCTAAGG AAGCTAAA**GCGAGGGCCTGAGGGCCATG**	pSLICER assembly primer ($DrCm^R$)
BK1946_R	GGCTTGATTTTCAGAATAGGGGCCAATCCAGA ATTACCTC**AAAAAACCCCCCGGATTGCC**	pSLICER assembly primer ($DrCm^R$)
BK1947_F	GCAGAAAAAATCCCCCCGGTGGCAATCCGGGG GGTTTTTT**GAGGTAATTCTGGATTGGCC**	pSLICER assembly primer (*I-Sce*I endonuclease)
BK1947_R	CGCTATAATGACCCCGAAGCAGGGTTATGCAG CGGAAGAT**GAGCAGAGGCTCTCGCTGAT**	pSLICER assembly primer (*I-Sce*I endonuclease)
BK1948_F	TGGCCCTCACCGCCGCGTCCATCAGCGAGAGC CTCTGCTC**ATCTTCCGCTGCATAACCCT**	pSLICER assembly primer (*oriT*)
BK1392_R	CGCCAGCCCAGCGGCGAGGGCAACCAGCTCGA TTAATTAA**GATCGTCTTGCCTTGCTCGT**	pSLICER assembly primer (*oriT*)
pSD1 (RM1) Assembly Primers		
BK1388_F	AGTACATCACCGACGAGCAAGGCAAGACGATC TTAATTAA**TCGAGCTGGTTGCCCTCGCC**	pSD1 assembly primer (split pCC1BAC-yeast #1)
BK1388_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAAC AGTTCTCA**TCCGGATCTGACCTTTACCA**	pSD1 assembly primer (split pCC1BAC-yeast #1)
BK1389_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCA GATCCGGA**TGAGAACTGTTTTTGAACAG**	pSD1 assembly primer (split pCC1BAC-yeast #2)
BK2093_R	CACGGCCGCGCTCGGCCTCTCTGGCGGCCTTCT GGCGCTC**GGGCTTCGCCCTGTCGCTCG**	pSD1 assembly primer (split pCC1BAC-yeast #2)
BK2094_F	CCAGTAGTGCTCGCCGCAGTCGAGCGACAGGG CGAAGCCC**GAGCGCCAGAAGGCCGCCAG**	pSD1 assembly primer (Tet^R)
BK2094_R	GAGCGCAATGCCCCGATCACCTTGCGGTACCG GGCGAGGC**GATCAGACGCTGAGTGCGCT**	pSD1 assembly primer (Tet^R)
BK2095_F	GCAGGACGCCGATGATTTGAAGCGCACTCAGC GTCTGATC**GCCTCGCCCGGTACCGCAAG**	pSD1 assembly primer (ORF14075 homology #1)
BK2095_R	AGGGCAGTTGGAAAGTTGAGGAAAGCAGGCG TGTGTACCAGGTGCCCCGCGACGTTGCGT**GTG CGCAGGAGTGGGCCACA**	pSD1 assembly primer (ORF14075 homology #1)
BK2096_F	TCCTCAACTTTCCAACTGCCCTCTCCGACTGAC	pSD1 assembly primer (Nm^R)

	TTTGCTGCTTAGGGATAACAGGGTAATGGGG TGGGCGAAGAACTCCA	
BK2096_R	TCTCATTTCACTAAATAATAGTGAACGGCAGG TATATGTGAGCTTCACGCTGCCGCAAGC	pSD1 assembly primer (Nm^R)
BK2097_F	GCAGCCCTTGCGCCCTGAGTGCTTGCGGCAGC GTGAAGCTCACATATACCTGCCGTTCAC	pSD1 assembly primer (*SacB*)
BK2097_R	AGGGCAGTTGGAAAGTTGAGGAAAGCAGGCG TGTGTACCAGGTGCCCCGCGACGTTGCGTGGC CATCGGCATTTTCTTTT	pSD1 assembly primer (*SacB*)
BK2098_F	TGGTACACACGCCTGCTTTCCTCAACTTTCCAA CTGCCCTCTCCGACTGACTTTGCTGCTAGCTTG AGATTCGTACTCGC	pSD1 assembly primer (ORF14075 homology #2)
BK2098_R	CGCTATAATGACCCCGAAGCAGGGTTATGCAG CGGAAGATCCCAGACCTGCTCCGGCGTG	pSD1 assembly primer (ORF14075 homology #2)
BK2099_F	CTGAAAAAAGCCCGCATCGGCACGCCGGAGCA GGTCTGGGATCTTCCGCTGCATAACCCT	pSD1 assembly primer (*oriT*)
BK1392_R	CGCCAGCCCAGCGGCGAGGGCAACCAGCTCGA TTAATTAAGATCGTCTTGCCTTGCTCGT	pSD1 assembly primer (*oriT*)
	pSD2 (RM2) Assembly Primers	
BK1388_F	AGTACATCACCGACGAGCAAGGCAAGACGATC TTAATTAATCGAGCTGGTTGCCCTCGCC	pSD2 assembly primer (split pCC1BAC-yeast #1)
BK1388_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAAC AGTTCTCATCCGGATCTGACCTTTACCA	pSD2 assembly primer (split pCC1BAC-yeast #1)
BK1389_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCA GATCCGGATGAGAACTGTTTTTGAACAG	pSD2 assembly primer (split pCC1BAC-yeast #2)
BK2299_R	ACAAGCATAAAGCTTGCTCAATCAATCACCGG ATCCCCGGGGGCTTCGCCCTGTCGCTCG	pSD2 assembly primer (split pCC1BAC-yeast #2)
BK2300_F	CCAGTAGTGCTCGCCGCAGTCGAGCGACAGGG CGAAGCCCCCGGGGATCCGGTGATTGAT	pSD2 assembly primer ($Spec^R$)
BK2300_R	CCAGCGGCTACGGGCGATGTACGAGCAGGAAC TGGGATTCGGATCCGGTGATTGATTGAG	pSD2 assembly primer ($Spec^R$)
BK2301_F	GTTTACAAGCATAAAGCTTGCTCAATCAATCA CCGGATCCGAATCCCAGTTCCTGCTCGT	pSD2 assembly primer (*Mrr* Homology #1)
BK2301_R	GCTGGAGTTCTTCGCCCACCCCATTACCCTGT TATCCCTAATTTTTTAAGTTTACGCTCT	pSD2 assembly primer (*Mrr* Homology #1)
BK2302_F	ACAGAGCGTAAACTTAAAAAATTAGGGATAA CAGGGTAATGGGGTGGGCGAAGAACTCCA	pSD2 assembly primer (Nm^R)
BK2302_R	TGTGAGCTAGCATTATACCTAGGACTGAGCTA GCTGTCAAAGCTTCACGCTGCCGCAAGC	pSD2 assembly primer (Nm^R)
BK2303_F	GCAGCCCTTGCGCCCTGAGTGCTTGCGGCAGC GTGAAGCTTTGACAGCTAGCTCAGTCCT	pSD2 assembly primer (*lacZ*)
BK2303_R	GTCTGGACTTACGGCTTTCGTCCCTTCCGCGCA CCCAGCGCCTGTCCCAGCGACGCCCGCTATAA ACGCAGAAAGGCCCA	pSD2 assembly primer (*lacZ*)
BK2304_F	CGCTGGGTGCGCGGAAGGGACGAAAGCCGTA AGTCCAGACAGAGCGTAAACTTAAAAAATGCT CGCCTGACAGGGCGGTT	pSD2 assembly primer (*Mrr* Homology #2)
BK2304_R	CGCTATAATGACCCCGAAGCAGGGTTATGCAG CGGAAGATCGGCAGTTCCACGTTGCACA	pSD2 assembly primer (*Mrr* Homology #2)
BK2305_F	CACCGCCCACATCGCCGAGTTGTGCAACGTGG AACTGCCGATCTTCCGCTGCATAACCCT	pSD2 assembly primer (*oriT*)

BK1392_R	CGCCAGCCCAGCGGCGAGGGCAACCAGCTCGATTAATTAA**GATCGTCTTGCCTTGCTCGT**	pSD2 assembly primer (*oriT*)
	pSD3 (RM3) Assembly Primers	
BK2390_F	CAAAGGCCTGCACGTCCTCAAAGAGCAGCGGCTGAATCAC**ATCTTCCGCTGCATAACCCT**	pSD3 assembly primer (*oriT* + split pCC1BAC-yeast #1)
BK2092_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAACAGTTCTCA**TCCGGATCTGACCTTTACCA**	pSD3 assembly primer (*oriT* + split pCC1BAC-yeast #1)
BK2093_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCAGATCCGGA**TGAGAACTGTTTTTGAACAG**	pSD3 assembly primer (split pCC1BAC-yeast #2 +Tet^R)
BK2391_R	GGGCCTACATAAAAGGATCAGTCCTCGGAAACTTCTGCCC**GATCAGACGCTGAGTGCGCT**	pSD3 assembly primer (split pCC1BAC-yeast #2 +Tet^R)
BK2392_F	GCAGGACGCCGATGATTTGAAGCGCACTCAGCGTCTGATC**GGGCAGAAGTTTCCGAGGAC**	pSD3 assembly primer (ORF15360 Homology #1)
BK2392_R	GCTGGAGTTCTTCGCCCACCCC**ATTACCCTGTTATCCCTA**ACAATGCCATTTATGTTTTC	pSD3 assembly primer (ORF15360 Homology #1)
BK2393_F	CAGAAAACATAAATGGCATTGT**TAGGGATAACAGGGTAAT**GGGGTGGGCGAAGAACTCCA	pSD3 assembly primer (Nm^R + *lacZ*)
BK2395_R	TGGCACTTCGGCACTAGCTGCGTCAGCCTTGTTTATTGACTCCGGCACGACTTGGAGACG**TATAAACGCAGAAAGGCCCA**	pSD3 assembly primer (Nm^R + *lacZ*)
BK2394_F	GTCAATAAACAAGGCTGACGCAGCTAGTGCCGAAGTGCCAGAAAACATAAATGGCATTGT**GCCCCCTCGACCTTGCCCGT**	pSD3 assembly primer (ORF15360 Homology #2)
BK2394_R	CGCTATAATGACCCCGAAGCAGGGTTATGCAGCGGAAGAT**GTGATTCAGCCGCTGCTCTT**	pSD3 assembly primer (ORF15360 Homology #2)
	pSD4 (RM4) Assembly Primers	
BK2408_F	GTTCGACCAAATGCGCCCCCCCACCCGCAGCGTCAGGTCG**ATCTTCCGCTGCATAACCCT**	pSD4 assembly primer (*oriT* + split pCC1BAC-yeast #1)
BK2092_R	GAAGAGCGTTGATCAATGGCCTGTTCAAAAACAGTTCTCA**TCCGGATCTGACCTTTACCA**	pSD4 assembly primer (*oriT* + split pCC1BAC-yeast #1)
BK2093_F	GTACGTGAAACGGATGAAGTTGGTAAAGGTCAGATCCGGA**TGAGAACTGTTTTTGAACAG**	pSD4 assembly primer (split pCC1BAC-yeast #2 +Tet^R)
BK2409_R	GCACACCCTGGTCGGCGCCGGGGCGACAGAGGGCGGCAGT**GATCAGACGCTGAGTGCGCT**	pSD4 assembly primer (split pCC1BAC-yeast #2 +Tet^R)
BK2410_F	GCAGGACGCCGATGATTTGAAGCGCACTCAGCGTCTGATC**ACTGCCGCCCTCTGTCGCCC**	pSD4 assembly primer (ORF15360 Homology #1)
BK2410_R	GCTGGAGTTCTTCGCCCACCCC**ATTACCCTGTTATCCCTA**TCGCCCACCTGATGATCGAG	pSD4 assembly primer (ORF15360 Homology #1)
BK2411_F	TACTCGATCATCAGGTGGGCGA**TAGGGATAACAGGGTAAT**GGGGTGGGCGAAGAACTCCA	pSD4 assembly primer (Nm^R + *lacZ*)
BK2413_R	TACGGCGTGGGCGTGCTGACCCGCGAGACCTACCAGATTCGCCGCTTAGACGCGGATTAT**TATAAACGCAGAAAGGCCCA**	pSD4 assembly primer (Nm^R + *lacZ*)
BK2412_F	GAATCTGGTAGGTCTCGCGGGTCAGCACGCCCACGCCGTACTCGATCATCAGGTGGGCGA**AATTCGGCCAGTCGCCGGTA**	pSD4 assembly primer (ORF15360 Homology #2)
BK2412_R	CGCTATAATGACCCCGAAGCAGGGTTATGCAGCGGAAGAT**CGACCTGACGCTGCGGGTGG**	pSD4 assembly primer (ORF15360 Homology #2)
	Seamless Deletion Cassette Amplification	
BK1965_F	CCGGTACCGCAAGGTGAT	RM1

BK1965_R	GGGTCGGTGTCCATCTCTT	RM1
BK1964_F	CCCAGTTCCTGCTCGTACAT	RM2
BK1964_R	AGTTCCACGTTGCACAACTC	RM2
BK1966_F	GCAGAAGTTTCCGAGGACTG	RM3
BK1966_R	GCTGCTCTTTGAGGACGTG	RM3
BK2378_F	GGTGTGCAGCTCGTCTATGA	RM4
BK2378_R	CGCATTTGGTCGAACAGC	RM4
Seamless Deletion Multiplex Primers		
BK2451_F	GAACGGGTGCAAATCAAGAC	Drad gDNA control – 150 bp
BK2451_R	CCGCGTCACCGAGTACAT	Drad gDNA control – 150 bp
BK2005_F	ACGACCATCACACCACTGAA	Plasmid backbone – 645 bp
BK2005_R	CATGACCAGCGTTTATGCAC	Plasmid backbone – 645 bp
BK2000_F	CGAAACGATCCTCATCCTGT	Nm marker – 311 bp
BK2000_R	AGGAAGCGGAACACGTAGAA	Nm marker – 311 bp
BK2003_F	GGCCCACTTCATCACAGAGT	RM1 (ORF14075) – 509 bp
BK2003_R	CCGAACAGGTCCTGGAAGTA	RM1 (ORF14075) – 509 bp
BK2216_F	CCTGACCGAAAGAGAGTTCG	RM2 (*Mrr*) – 508 bp
BK2216_R	GTAGCGGGAGGTCGTCATAA	RM2 (*Mrr*) – 508 bp
BK2004_F	GCTGGTAAATGCCCTTCGTA	RM3 (ORF15360) – 510 bp
BK2004_R	TCTACGCCGACTTCCTGTTC	RM3 (ORF15360) – 510 bp
BK2450_F	CTGAACCCGGACGTAGTGAT	RM4 (Mrr2) – 460 bp
BK2450_R	TTCAGACCATTCCGGCTTAC	RM4 (Mrr2) – 460 bp

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1. House BL, Mortimer MW, Kahn ML. New recombination methods for Sinorhizobium meliloti genetics. Appl Environ Microbiol. 2004;

2. Brumwell SL, Van Belois KD, Giguere DJ, Edgell DR, Karas BJ. Conjugation-Based Genome Engineering in Deinococcus radiodurans. ACS Synth Biol. 2022;11(3).

3. Meima R, Lidstrom ME. Characterization of the minimal replicon of a cryptic Deinococcus radiodurans SARK plasmid and development of versatile Escherichia coli-D. radiodurans shuttle vectors. Appl Environ Microbiol. 2000;66(9):3856–67.

4. Strand TA, Lale R, Degnes KF, Lando M, Valla S. A New and Improved Host-Independent Plasmid System for RK2-Based Conjugal Transfer. PLoS One. 2014 Mar;9(3):e90372.

Curriculum Vitae

STEPHANIE BRUMWELL

Department of Biochemistry PhD Candidate

University of Western Ontario London, Ontario N6A 5C1

EDUCATION

PhD Candidate, Biochemistry 2016 – Present
University of Western Ontario, London, ON

Bachelor of Science, Honours Biology, Co-operative Program 2011 – 2016
University of Waterloo, Waterloo, ON
Dean's Honours List

SCHOLARSHIPS AND AWARDS

Ontario Graduate Scholarship	$15,000	2020
Department of Biochemistry Best Poster	$50	2020
Ontario Graduate Scholarship	$15,000	2019
2nd place oral presentation, Synthetic Biology Symposium 4.0	$100	2019
3rd place poster presentation, Synthetic Biology Symposium 2.0	$50	2017
Faculty of Science Entrance Scholarship, University of Waterloo	$500	2011

PUBLICATIONS

Brumwell, S.L., Van Belois, K.D., Edgell, D.R., Karas, B.K. (2022) SLICER: seamless loss of integrated cassettes using endonuclease cleavage and recombination in *Deinococcus radiodurans*. *ACS Synthetic Biology*, In Preparation.

Koehle, A.P., **Brumwell, S.L.**, Seto, E.P., Lynch, A.M., Urbaniak, C. (2022) The importance of microbes in future space missions to the Moon, Mars and beyond. *Frontiers in Microbiology*, In Preparation.

Cochrane, R.R., Shrestha, A., Severo de Almeida, M.M., Agyare-Tabbi, M., **Brumwell, S.L.**, … Karas, B.J. (2022) Superior conjugative plasmids delivered by bacteria to diverse fungi. *BioDesign*, In Review, Manuscript # BIODESIGNR-D-22-00014.

Brumwell, S.L., Van Belois, K.D., Giguere, D.J., Edgell, D.R. and Karas, B.J. (2022) Conjugation-based genome engineering in *Deinococcus radiodurans*. *ACS Synthetic Biology*, 11(3), 1068-1076.

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.

Yoshida, C., **Brumwell, S.L.**, Lingohr, E.J., Ahmad, A., Blimkie, T.M., … Nash, J.H.E. (2016) Draft whole-genome sequences of 25 *Salmonella enterica* strains representing 24 serovars. *Genome announcements*, *4*(2): e01718-15.

PRESENTATIONS

A new bacterial chassis for *rad* synthetic biology. Graduate Research Symposium, Western University, London, ON, Canada. December, 2020 (Poster).

Development of bacterial hosts for agricultural and biotech applications. Graduate Research Symposium, Western University, London, ON, Canada. November, 2019 (Talk).

Designer *Sinorhizobium meliloti* strains enable direct trans-kingdom DNA transfer. Synthetic Biology Symposium 4.0, Waterloo, ON, Canada. May, 2019 (Talk).

Development of *Sinorhizobium meliloti* as a host for cloning, storage and delivery of DNA to prokaryotic and eukaryotic microorganisms. Canadian Society of Microbiologist 69th Annual Conference, Sherbrooke, QC, Canada. June, 2019 (Poster).

Development of *Sinorhizobium meliloti* as a host for cloning, storage and delivery of DNA to prokaryotic and eukaryotic microorganisms. Synthetic Biology: Engineering, Evolution & Design (SEED), Scottsdale, AZ, USA. June, 2018 (Poster).

Development of *Sinorhizobium meliloti* as a host for cloning, storage and delivery of DNA to prokaryotic and eukaryotic microorganisms. Synthetic Biology Symposium 3.0, London, ON, Canada. May, 2018 (Talk and Poster).

Development of an advanced genetic toolbox to enable genome scale engineering in *Sinorhizobium meliloti*. (2017) Canadian Society of Microbiologist 67th Annual Conference, Waterloo, ON, Canada. June, 2017 (Poster).

Development of an advanced genetic toolbox to enable genome scale engineering in *Sinorhizobium meliloti*. Synthetic Biology Symposium 2.0, London, ON, Canada. May, 2017 (Poster).

Development of an advanced genetic toolbox to enable genome scale engineering in *Sinorhizobium meliloti*. London Health Research Day, London, ON. March, 2017 (Poster).

TEACHING AND MENTORING EXPERIENCE

Teaching Assistantship, University of Western Ontario

Biochemistry 3392F/G: Synthetic Biology	2020
Biochemistry 3380G: Biochemistry Laboratory	2019
Biochemistry 2288A: Biochem and Molecular Biology for Food and Nutrition	2017

Undergraduate Mentorship, University of Western Ontario

Katherine Van Belois	3rd year intern; 4th year Biology thesis student	2020 – 2022
Joanna Rossa	2nd year volunteer and work study student	2019 – 2020
Jordyn Meaney	4th year Biology thesis student	2018 – 2019
Kaitlyn Dan	3rd year volunteer and work study student	2018 – 2019
Michael Groff	3rd year volunteer and work study student	2018 – 2019
Jack Whelan	4th year Biochemistry thesis student	2017 – 2018
Tony Huang	3rd year Biochemistry project student	2017 – 2018
Western Synthetic Biology Research Program (WSBR)		2016 – 2019

LEADERSHIP AND EXTRACURRICULAR ACTIVITIES

Biochemistry Department EDI Workshop and Committee, Organizer (UWO)	2021
Edmonton Women in Tech Day, Invited Speaker - Q&A (Virtual)	2021
Edmonton Women in Tech Day, Invited Speaker - Panel (Edmonton, AB)	2020
Forest City SynBio Programs Committee Chair (London, ON)	2019 – 2021
Biochemistry Graduate Student Association Member (UWO)	2019 – 2021
Society of Graduate Students Finance Committee Member (UWO)	2019 – 2021
Biochemistry Gender Equity Task Force (UWO)	2018 – 2019
Synthetic Biology Symposium 2.0 and 3.0 Volunteer (UWO)	2017 – 2018

RELEVANT WORK EXPERIENCE

Research Lab Assistant — May – Sept 2016
Dr. Bruce Reed's Lab, Department of Biology, University of Waterloo, Waterloo, ON

Bioinformatics Research Assistant — Sept – Dec 2013, Jan – Sept 2015
National Microbiology Laboratory, Public Health Agency of Canada, Guelph, ON

Field and Lab Assistant — Jan – Apr 2013
Environmental Geochemistry Laboratory, Department of Earth & Environmental Sciences, University of Waterloo, Waterloo, ON