Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 5212; doi:10.3390/ijms20205212

*Article*

# Trans-Kingdom Conjugation Within Solid Media from *Escherichia coli* to *Saccharomyces cerevisiae*

**Maximillian P.M. Soltysiak [1], Rebecca S. Meaney [2,3,†], Samir Hamadache [1,3,†], Preetam Janakirama [2], David R. Edgell [3] and Bogumil J. Karas [2,3,*]**

[1] Department of Biology, The University of Western Ontario, London, N6A 5C1, Ontario, Canada; msoltys4@uwo.ca, shamadac@uwo.ca
[2] Designer Microbes Inc., London, Canada; rmeaney2@uwo.ca, preetam.janakirama@gmail.com, bkaras@uwo.ca
[3] Department of Biochemistry, Schulich School of Medicine and Dentistry, University of Western Ontario, London, N6A 5C1, Ontario, Canada; rmeaney2@uwo.ca, shamadac@uwo.ca, dedgell@uwo.ca, bkaras@uwo.ca
* Correspondence: bkaras@uwo.ca

**Abstract:** Conjugation is a bacterial mechanism for DNA transfer from a donor cell to a wide range of recipients, including both prokaryotic and eukaryotic cells. In contrast to conventional DNA delivery techniques, such as electroporation and chemical transformation, conjugation eliminates the need for DNA extraction, thereby preventing DNA damage during isolation. While most established conjugation protocols allow for DNA transfer in liquid media or on a solid surface, we developed a procedure for conjugation within solid media. Such a protocol may expand conjugation as a tool for DNA transfer to species that require semi-solid or solid media for growth. Conjugation within solid media could also provide a more stable microenvironment in which the conjugative pilus can establish and maintain contact with recipient cells for the successful delivery of plasmid DNA. Furthermore, transfer in solid media may enhance the ability to transfer plasmids and chromosomes greater than 100 kbp. Using our optimized method, plasmids of varying sizes were tested for transfer from *E. coli* to *S. cerevisiae*. We demonstrated that there was no substantial decrease in conjugation frequency as plasmid size increased—up to 138.5 kbp in length. Finally, we established an efficient PCR-based synthesis protocol to generate custom conjugative plasmids.

**Keywords:** conjugation; solid media; *Saccharomyces cerevisiae*; Trans-Kingdom; *Escherichia coli*; pTA-Mob; yeast assembly

## 1. Introduction

Conjugation is a widespread bacterial mechanism for DNA transfer and a major contributor to the spread of antibiotic resistance and virulence factors [1]. Through the advent of recombinant DNA technology, conjugation has been adapted for extensive use in biotechnology as a simple alternative for DNA transfer to a broad range of recipient species. Although initially described as a prokaryotic phenomenon, conjugal transfer is not limited strictly to bacterial recipients. Trans-kingdom conjugation is observed in nature in the form of T-DNA transfer from *Agrobacterium* species to plants [2,3]. For bioengineering purposes, various bacterial donor species have been used to deliver DNA to eukaryotic recipients such as the yeast *Saccharomyces cerevisiae* [4–8], algal diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* [8–12], and mammalian cells [13–16].

Conventional transformation techniques, such as electroporation and chemical transformation [17], have been developed for many species, yet suffer from some drawbacks [18]. For one, these methods require pure, intact DNA molecules, which can be challenging since large molecules (>100

kbp) are prone to damage from shear forces during purification and handling [19]. Conjugation provides an alternative approach to achieve delivery of either self-transferring (*cis*) or mobilizable (*trans*) plasmids into a wide range of recipient species. For *in situ* applications, conjugation is especially useful when conventional transformation techniques can be difficult or even impossible to use, such as in DNA delivery to soil rhizospheres [20,21] or gut microbiomes [22]. Although easy to use when transferring DNA between prokaryotic cells, optimal conditions for conjugal transfer to eukaryotes remain poorly explored. Furthermore, while it has been previously demonstrated that plasmids up to 875 kbp in size can be transferred to prokaryotic recipients, the upper size limit of conjugal transfer to eukaryotes has yet to be determined [23].

The majority of conjugation systems use a subfamily of the type IV secretion system (T4SS) to export DNA to recipient cells; however, the composition and structure of T4SS complexes differ among the identified conjugative plasmid groups [24,25]. Some conjugation systems, such as IncF, IncH, and IncI plasmids, transfer DNA more efficiently in liquid media, while others, including the IncN, IncM, IncP, and IncW plasmids, achieve higher DNA transfer frequencies on solid media [26]. It is suspected that the ability to transfer DNA in different environmental conditions is related to variation in pilus formation, structure, and stability of cells during the conjugation process. If conjugation would occur within solid media—as opposed to on the surface or in liquid—the environment may be more stable. The additional stability could prolong the time a donor bacterium is attached to the recipient cell, and therefore, may be more conducive to transfer of large plasmids or even whole chromosomes. A protocol where conjugal transfer occurs within solid media may also expand the use of conjugation as a tool for DNA transfer to species that require semi-solid or solid media for growth [27–29]. Furthermore, such a protocol may permit more accurate enumeration of transconjugants by avoiding the use of a spreader while plating cells on selective media [30].

We developed and optimized a simple protocol for *cis* and *trans* conjugal transfer from *E. coli* to *S. cerevisiae* within solid media. Using the newly developed solid media conjugation protocol, we transferred plasmids to *S. cerevisiae* ranging in size from 18.1 kbp to 138.5 kbp. Notably, we showed that there was no substantial decrease in conjugation frequency as the size of the plasmids increased. We also established an efficient and reproducible PCR-based synthesis pipeline to generate the conjugative plasmid pTA-Mob 2.0, a derivative of the IncP plasmid pTA-Mob [31]. Both tools improve how we build and deliver DNA via conjugation from prokaryotic to eukaryotic cells.

## 2. Materials and Methods

### *2.1. Strains and growth conditions*

NEB 5-alpha Electrocompetent *Escherichia coli* (New England Biolabs Ltd., #C2987) and Transformax Epi300 Electrocompetent *E. coli* (Lucigen, #EC300110) were grown in Luria-Bertani (LB) media supplemented with the appropriate antibiotic(s): chloramphenicol (30 μg/mL) and/or gentamicin (40 μg/mL). *Saccharomyces cerevisiae* VL6-48 (ATCC no. MYA-3666) was grown in 2x yeast extract/peptone/dextrose media (YPD) supplemented with 200 μg/mL adenine hemisulfate (Sigma-Aldrich, #A2545)(YPDA) and 100 μg/mL ampicillin. For yeast spheroplast transformation/plasmid assembly, complete minimal (CM) glucose media lacking histidine and uracil (Teknova, #C7221) supplemented with adenine hemisulfate (100 μg/mL) and 1 M D-sorbitol. For yeast conjugation plates, either CM glucose media lacking histidine supplemented with 60 μg/mL adenine (Teknova, #C7112) or CM glucose media lacking histidine and uracil supplemented with adenine hemisulfate (100 μg/mL) was used when appropriate.

### *2.2. Construction of pTA-Mob 2.0 plasmid*

The first iteration the pTA-Mob 2.0 plasmid was generated by PCR amplification of pTA-Mob [31]in nine overlapping fragments (D501F/R – D509F/R); amplification of *HIS3-CEN6-ARS4* (D510F/R) and *URA3* (D512F/R)from a Designer Microbes Inc. plasmid (pDMI-1.0, unpublished); and

the RK2/RP4 origin of transfer sequence (*oriT*) (D511F/R) from p0521s [9]. In the final optimized protocol, the pTA-Mob 2.0 plasmid was generated by amplifying the vector as ten overlapping fragments using primers (Fragment_1_F/R – Fragment_10_F/R) listed in Supplementary Table 1.

Each fragment was individually amplified in a 50 µL PCR reaction using PrimeSTAR GXL polymerase (Takara Bio Inc., #R050A), 1 µL of template DNA (see specific concentrations below), and the respective forward and reverse primers in a final concentration of 0.2 µM. For the final optimized protocol, 1 µL of 10 ng/µL template plasmid was used for fragments 1–6 and 8–10, while 1 µL of 50 ng/µL template plasmid was used for fragment 7 due to poor initial amplification. (Note: For the initial 12 fragment assembly each fragment was amplified using 1–2 ng/µL of template DNA.) The PCR programming for the optimized protocol was as follows: 25 cycles of 98°C for 10 seconds, 61°C for 15 seconds, and 68°C for 70 seconds, followed by 1 cycle of 68°C for 60 seconds, ending with an infinite hold at 12°C. Amplification was confirmed using agarose gel electrophoresis by runnning 1 µL of PCR product on a 1.4% agarose (w/v) gel.

To eliminate the template DNA from the PCR products, each reaction was treated with 10 units (0.5 µL) of DpnI restriction endonuclease (New England Biolabs Ltd., #R0176), incubated at 37°C for 30 minutes, and deactivated for 20 minutes at 80°C. Fragments were then purified from each solution using the EZ-10 Spin Column PCR Products Purification Kit (BioBasic Inc., #BS363) and diluted to approximately 60 ng/µL in nuclease-free water. Equimolar quantities of each of the ten purified fragments were mixed into a single 1.5-mL microcentrifuge tube (with a total volume of 20.4 µL). For negative controls, partial assembly mixes containing either fragment 1, fragment 2, or fragment 3 were used.

The purified fragments were assembled using yeast spheroplast transformation as described in Karas et al. [32] with the exception that the bacterial culture was replaced by mixtures of DNA fragments were used. Following the polyethylene glycol treatment and recovery, 100 µL of transformed spheroplasts were added to 8 mL of molten CM glucose media lacking histidine and uracil supplemented with adenine hemisulfate and with 1 M D-sorbitol and 2% agar. After mixing the cells by inversion, the media was poured directly into a Petri dish. Plates were incubated at 30°C for 24 hours prior to the addition of 8 mL of liquid CM glucose media lacking histidine and uracil to pool the transformed *S. cerevisiae* colonies. After an additional incubation for three days at 30°C, the liquid layer was transferred to a 15 mL centrifuge tube. Plasmid isolation was then carried out according to Karas et al. [9] and pelleted DNA was resuspended in 50 µL of elution buffer (Qiagen, #19086).

For the initial 12-fragment assembly, 1 µL of isolated pooled yeast DNA was added to 30 µL of NEB 5-alpha electrocompetent *E. coli* cells in a 1.5 mL microcentrifuge tube on ice. The mixture was transferred to a cold 1 mm electroporation cuvette and electroporated at 1.8 kV using the BioRad GenePulser. For the final optimized protocol, 25 µL of TransforMax Epi300 Electrocompetent *E. coli* cells were mixed with 1 µL of isolated pooled yeast DNA on ice. The mixture was transferred to a cold 2 mm electroporation cuvette and electroporated at 2.5 kV. Cells were recovered in 1 mL of Super Optimal broth with Catabolite repression [33] at 37°C, shaking at 225 rpm for 1 hour. Following recovery, 100 µL of the transformed cell mixture was plated on 1.5% agar (w/v) LB media plates containing gentamicin (40 µg/mL) and incubated at 37°C overnight.

To screen for correctly assembled plasmids, individual *E. coli* colonies were tested as follows:

For the initial 12-fragment assembly, 50 *E. coli* pTA-Mob 2.0 colonies were streaked onto a fresh LB agar plate containing gentamicin (40 µg/mL) and incubated at 37°C overnight. A second LB agar plate supplemented with chloramphenicol (30 µg/mL) was streaked with Epi300 *E. coli* containing the pAGE2.0 plasmid and incubated at 37°C overnight [8]. The next morning, each *E. coli* pTA-Mob 2.0 colony was streaked on top of *E. coli* pAGE2.0 on non-selective LB agar plates and incubated for

3 hours at 37°C. Cells were scraped and resuspended in liquid LB media before performing 10-fold serial dilutions from $10^{-1}$ to $10^{-4}$. Then, 5 µL of the serial dilutions for each sample were spot-plated on LB agar plates containing gentamicin (40 µg/mL) and chloramphenicol (30 µg/mL) and incubated at 37°C overnight. The ability of each *E. coli* colony to conjugate was assessed by transconjugant colony growth at each dilution the following day. One clone was selected, and the plasmid isolated from this strain was named pTA-Mob 2.0. Plasmid DNA was isolated using the EZ-10 Spin Column Plasmid DNA Miniprep Kit (BioBasic Inc., #BS413) and sent for Complete Plasmid Sequencing at the Massachusetts General Hospital DNA Core.

For the final optimized plasmid assembly protocol, 30 colonies were tested. First, the colonies were streaked on LB plates supplemented with gentamicin (40 µg/mL) and incubated at 37°C overnight. A second LB agar plate containing chloramphenicol (30 µg/mL) was streaked with Epi300 *E. coli* containing the pAGE1.0 plasmid and incubated at 37°C overnight [8]. The next day, overnight cultures of 3 mL LB supplemented with gentamicin (40 µg/mL) were inoculated with one of the 30 newly assembled donor *E. coli* pTA-Mob 2.0 colonies. In addition, two overnight cultures of recipient *E. coli* pAGE1.0 were grown in 3 mL LB containing chloramphenicol (30 µg/mL). As a positive control for conjugation, a 3 mL LB with gentamicin (40 µg/mL) culture of the original *E. coli* pTA-Mob 2.0 was also inoculated. Cultures were incubated overnight at 37°C in a tube rotator. After 16 hours, 100 µL of donor and 100 µL of recipient cultures were mixed, spread on non-selective LB agar plates, and incubated for 1 hour at 37°C. The following controls were also performed: i) original donor *E. coli* pTA-Mob 2.0 with recipient *E. coli* pAGE1.0 (positive control); ii) original donor *E. coli* pTA-Mob 2.0 with water (negative control), and; iii) recipient *E. coli* pAGE1.0 with water (negative control). Conjugation plates were then scraped with 1 mL LB media, and cells were transferred to a 1.5 mL microcentrifuge tube. Each mixture was serially diluted 10-fold from $10^{-1}$ to $10^{-5}$ using LB media, and a 5 µL volume of each of the serial dilutions was spot-plated onto LB agar plates supplemented with gentamicin (40 µg/mL) and chloramphenicol (30 µg/mL). Plates were incubated overnight at 37°C and photographed the next day.

### *2.3. Conjugation within solid media*

#### 2.3.1. Preparation of *E. coli*

Cultures of 50 mL of LB media supplemented with the appropriate antibiotic (40 µg/mL gentamicin for *E. coli* pTA-Mob 2.0 or 40 µg/mL gentamicin and 30 µg/mL chloramphenicol for *E. coli* pAGE1.0 [8] with pTA-Mob [31] and *E. coli* pBK-RBYV-25-2 [9,34] with pTA-Mob) were inoculated with freshly grown *E. coli* and incubated overnight at 37°C and 225 rpm in 250 mL Erlenmeyer flasks. The following morning, saturated bacterial cultures were diluted 100x to a total volume of 50 mL in LB media with the appropriate antibiotic in a new flask. The diluted cultures were grown to an optical density at 600 nm ($OD_{600}$) of 0.5, 1.0, or 2.0. The cultures were then transferred to 50 mL centrifuge tubes and pelleted for 10 minutes at 5,000 relative centrifugal force (rcf). After centrifugation, the supernatants were decanted, and the pellets were resuspended in 1 mL LB media.

#### 2.3.2. Preparation of *S. cerevisiae* VL6-48

A 50 mL overnight culture was inoculated with fresh yeast and grown at 30°C at 225 rpm in 2x YPDA media supplemented with ampicillin (100 µg/mL) in a 250 mL Erlenmeyer flask. The culture was grown to an $OD_{600}$ of 1.0. The culture was then transferred to a 50 mL centrifuge tube and pelleted for 10 minutes at 5,000 rcf. After decanting the supernatant, the cell pellet was resuspended in 1 mL sterile double deionized $H_2O$ (sdd$H_2O$).

#### 2.3.3. Conjugation in solid media procedure

Mixtures of 25, 50, or 100 µL of *E. coli* cell suspension with 50, 100, or 200 µL of *S. cerevisiae* cell suspension were brought to a final volume of 1 mL with sdd$H_2O$ in a 1.5 mL microcentrifuge tube. Centrifuge tubes containing 5 mL of either molten CM glucose media lacking histidine and uracil with 1%, 1.5%, or 2% agar (w/v) or molten CM glucose media lacking histidine supplemented with adenine with 2% agar were prepared and held in a water bath set to either 55°C, 60°C, or 65°C. The tubes were removed from the water bath, the cell mixture was added to the molten media and inverted three times to mix. Cells resuspended in agar were then poured onto the respective base plate of 25 mL CM glucose media lacking histidine and uracil with 2% agar or CM glucose media lacking histidine with 2% agar preincubated at 37°C. After a 20-minute drying period, conjugation plates were incubated at 30°C. Successful transconjugant *S. cerevisiae* colonies were then counted under 5x magnification using the Zeiss Discovery.V8 SteREO microscope at the Biotron Experimental Climate Change Research Centre at the University of Western Ontario.

To quantify conjugation frequency, the colony-forming units for the recipient *S. cerevisiae* were obtained by creating serial dilutions and plating 100 µL of diluted cells onto YPDA 1% agar plates. The *S. cerevisiae* plates were dried and then incubated for two days at 30°C prior to counting colonies.

#### 2.3.4. Confirmation of successfully transferred plasmids

After the four-day incubation period, colony yields were counted. Single colonies were picked from the agar and streaked onto CM glucose media lacking histidine with 2% agar plates containing ampicillin (100 µg/mL). A total of three successive passages were performed, streaking a small portion of cells after a two-day incubation at 30°C each time. Following the second passage, a small portion of cells were streaked onto LB plates containing gentamicin (40 µg/mL) to test for surviving donor *E. coli*. After the third passage, *S. cerevisiae* colony streaks were resuspended in 100 µL TE buffer (pH 8). Cell resuspensions were then lysed at 98°C for 15 minutes. Multiplex PCR was performed using Qiagen Multiplex PCR Kit (Qiagen, #206143) using the cell lysate as template for primers (Supplementary Table 1) that bind to pAGE1.0, pTA-Mob 2.0, and pBK-RBYV-25-2 in locations distributed around each plasmid. The Multiplex PCR conditions were as follows: a 95°C hot start for 15 minutes, 35 cycles of 94°C for 30 seconds, 60°C for 90 seconds, and 72°C for 45 seconds, followed by a final extension at 72°C for 10 minutes, and a 12°C infinite hold. Multiplex amplification was confirmed using agarose gel electrophoresis with 2 µL of each Multiplex PCR reaction run on a 2% agarose (w/v) gel.

## 3. Results

### *3.1. PCR-based synthesis of conjugative plasmid pTA-Mob 2.0*

In previous studies, the non-mobilizable helper plasmid pTA-Mob was used to transfer destination plasmids from bacteria to recipient bacteria [31], algae [9,11,12], and yeast [8]. The pTA-Mob plasmid encodes the machinery required for conjugal transfer of vectors that contain an origin of transfer (*oriT*) [31]. For this study and future applications, we designed a method to build an alternative version of pTA-Mob, named pTA-Mob 2.0, that can self-mobilize (*cis*) and replicate in both *E. coli* and *S. cerevisiae*. To this end, we used PCR to initially amplify pTA-Mob as nine fragments along with three additional fragments: the *S. cerevisiae URA3* gene; a gene cassette containing the *S. cerevisiae HIS3* gene, yeast centromere (*CEN6*), and an autonomously replicating sequence (*ARS4*); and an *oriT* cassette. After assembling the fragments into whole plasmids in yeast, we transferred the DNA to *E. coli* and tested individual colonies for conjugation on top of agar to recipient *E. coli*. For subsequent work we selected one colony harboring pTA-Mob 2.0 and isolated the plasmid for optimization of a simplified PCR-based plasmid synthesis pipeline. The reason for this optimization step was to simplify the assembly from twelve to ten fragments as well as to obtain cleaner PCR products. This improved protocol functions to accelerate the creation of designer pTA-Mob 2.0 variants. We designed new primers to amplify the vector as ten approximately equal-sized

overlapping fragments (Figure 1a, b). Once again, following yeast assembly, we transferred DNA to *E. coli* and tested for conjugation to a recipient *E. coli* strain. This initial *E. coli* to *E. coli* conjugation analysis provided a rapid approach to evaluate successfully assembled plasmids before testing conjugal transfer to eukaryotic cells. Of the colonies tested, 23 out of the 30 donor colonies conjugated at least as well as the parental pTA-Mob 2.0 (Figure 1c, Supplementary Figure 1).

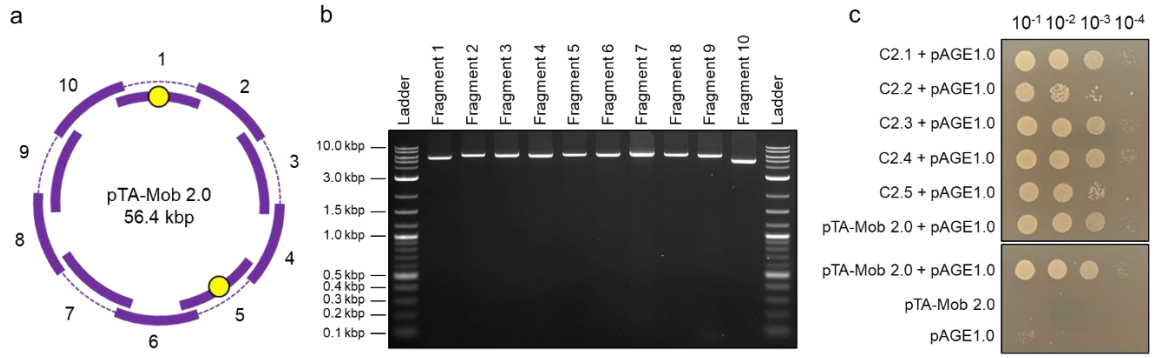


**Figure 1.** Optimized PCR-based synthesis of plasmid pTA-Mob 2.0: (**a**) Schematic of pTA-Mob 2.0 split into ten overlapping fragments. Yellow circles indicate positions of the yeast selection markers *HIS3* in fragment 1 and *URA3* in fragment 5; (**b**) Gel electrophoresis of pTA-Mob 2.0 amplified as ten overlapping fragments; (**c**) Partial conjugation results of newly assembled pTA-Mob 2.0 plasmids (colonies C2.1 to C2.5) to recipient *E. coli* containing the pAGE1.0 plasmid.

### *3.2. Development and optimization of conjugation within solid media*

The initial protocol for conjugation in solid media was inspired by the method for direct transfer of DNA from bacteria to yeast, where a polyethylene glycol-treated mixture of bacteria and yeast spheroplasts is suspended in molten agar media and then plated in a Petri dish [19,35]. For the development of the conjugation protocol, we used intact yeast cells and did not treat the mixture with polyethylene glycol. Early attempts performing the conjugation protocol resulted in inconsistent results (data not shown) prompting us to develop an optimized protocol. Optimization parameters included: i) molten agar media temperature, ii) molten agar media agar concentration, and iii) volumes of *E. coli* and *S. cerevisiae* cell suspensions harvested at various optical densities (ODs). We used the optimal value for each parameter in subsequent optimization experiments.

First, when testing the effect of molten agar media temperature, we found that a temperature of 60°C was optimal for transconjugant colony formation (Figure 2a). For the initial agar concentration, 2% agar (w/v) was the most conducive to colony formation (Figure 2b). Higher agar concentrations were not practical due to the rapid solidification of the media. We then tested 27 combinations of various volumes of donor and recipient cells harvested at different ODs and counted the number of yeast colonies per plate (Figure 2c). Based on this experiment, mixing 100 µL of donor and recipient cell suspensions, harvested at an $OD_{600}$ of 1.0, resulted in the highest number of transconjugant colonies.

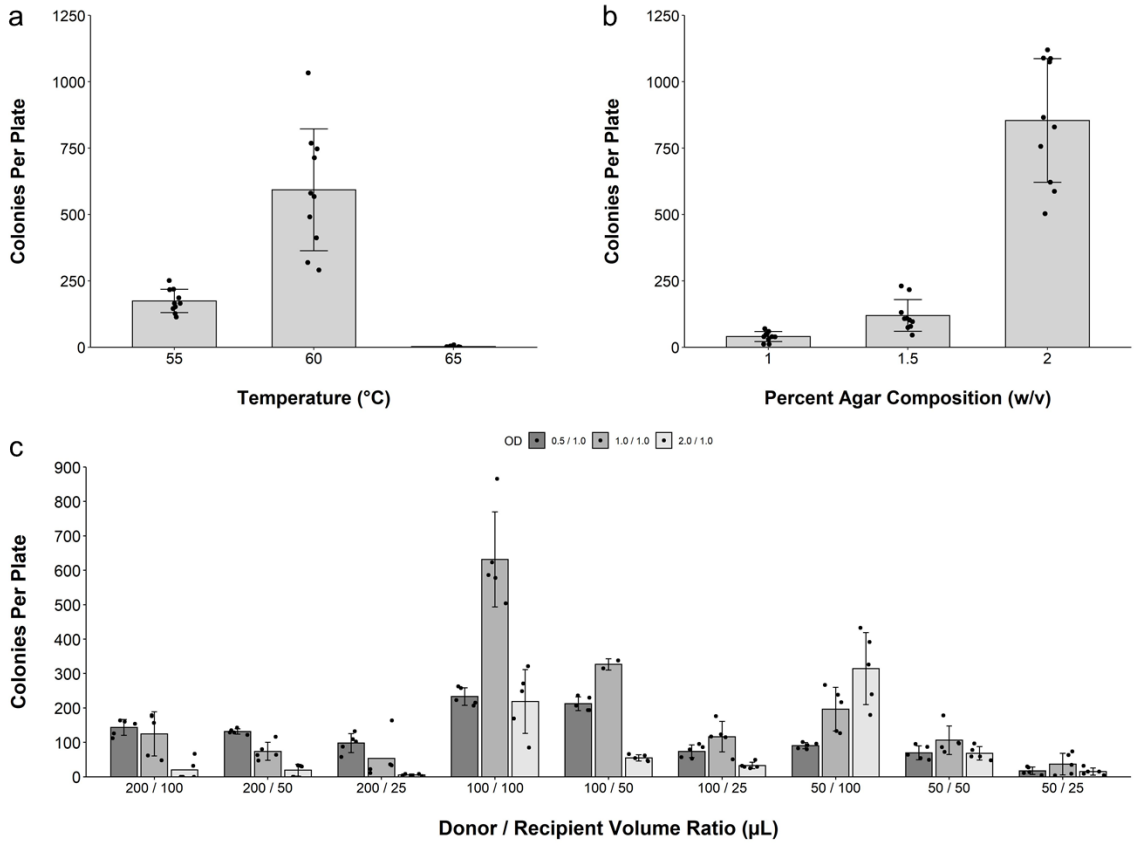


**Figure 2.** Optimization of the conjugation protocol in solid media. Colonies per plate yielded from optimization experiments testing: (**a**) Molten agar media temperature; (**b**) Molten agar media agar composition prior to the addition of cell mixture; (**c**) Volumes of *E. coli* and *S. cerevisiae* cell suspensions harvested at various optical densities ($OD_{600}$).

We summarized the optimization results in a final protocol for the conjugation in solid media method, as illustrated in Figure 3a, Supplementary Note 1. Using this optimized protocol, the highest number of transconjugants are obtained however the final colonies appear small (Figure 3b). Using smaller volumes of the final mix (100 µL/100 µL donor and recipient cell) such as 60% or 20%, fewer but larger colonies can be obtained (Figure 3b).

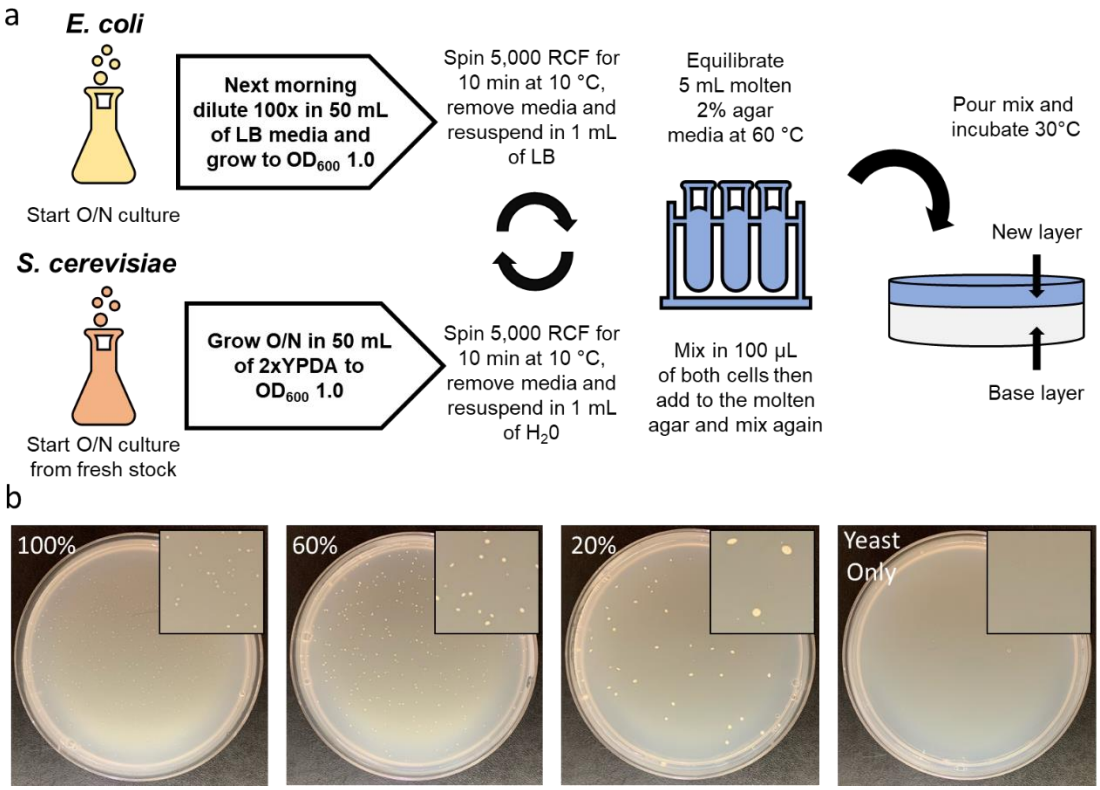


**Figure 3.** Optimized protocol for conjugation in solid media: (**a**) Schematic for the final protocol for conjugation from donor *E. coli* to recipient *S. cerevisiae* within solid media; (b) Comparison of using 100%, 60%, and 20% of the optimized 100 µL/100 µL donor and recipient cell ratio.

### *3.3. Conjugation of IncP-based vectors of increasing size*

Using the optimized protocol, we tested conjugation of plasmids of increasing size: pAGE1.0 (18.1 Kbp) [8], pTA-Mob 2.0 (56.4 Kbp), and pBK-RBYV-25-2 (138.5 Kbp) [9,34]. To transfer pAGE1.0 and pBK-RBYV-25-2, the donor *E. coli* also contained the pTA-Mob helper plasmid to allow for mobilization the plasmids in *trans* [31]. Surprisingly, conjugation with the larger plasmids produced more yeast colonies than with the smaller pAGE1.0 plasmid. Conjugation frequencies were then calculated using the number of transconjugant colonies divided by the colony-forming units obtained from the recipient *S. cerevisiae* serial dilutions. The conjugation frequencies for pAGE1.0, pTA-Mob 2.0, and pBK-RBYV-25-2 to *S. cerevisiae* were determined to be $1.9 \times 10^{-6}$, $3.3 \times 10^{-5}$, and $3.1 \times 10^{-5}$, respectively (Figure 4a).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 30 September 2019 doi:10.20944/preprints201909.0340.v1

Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 5212; doi:10.3390/ijms20205212



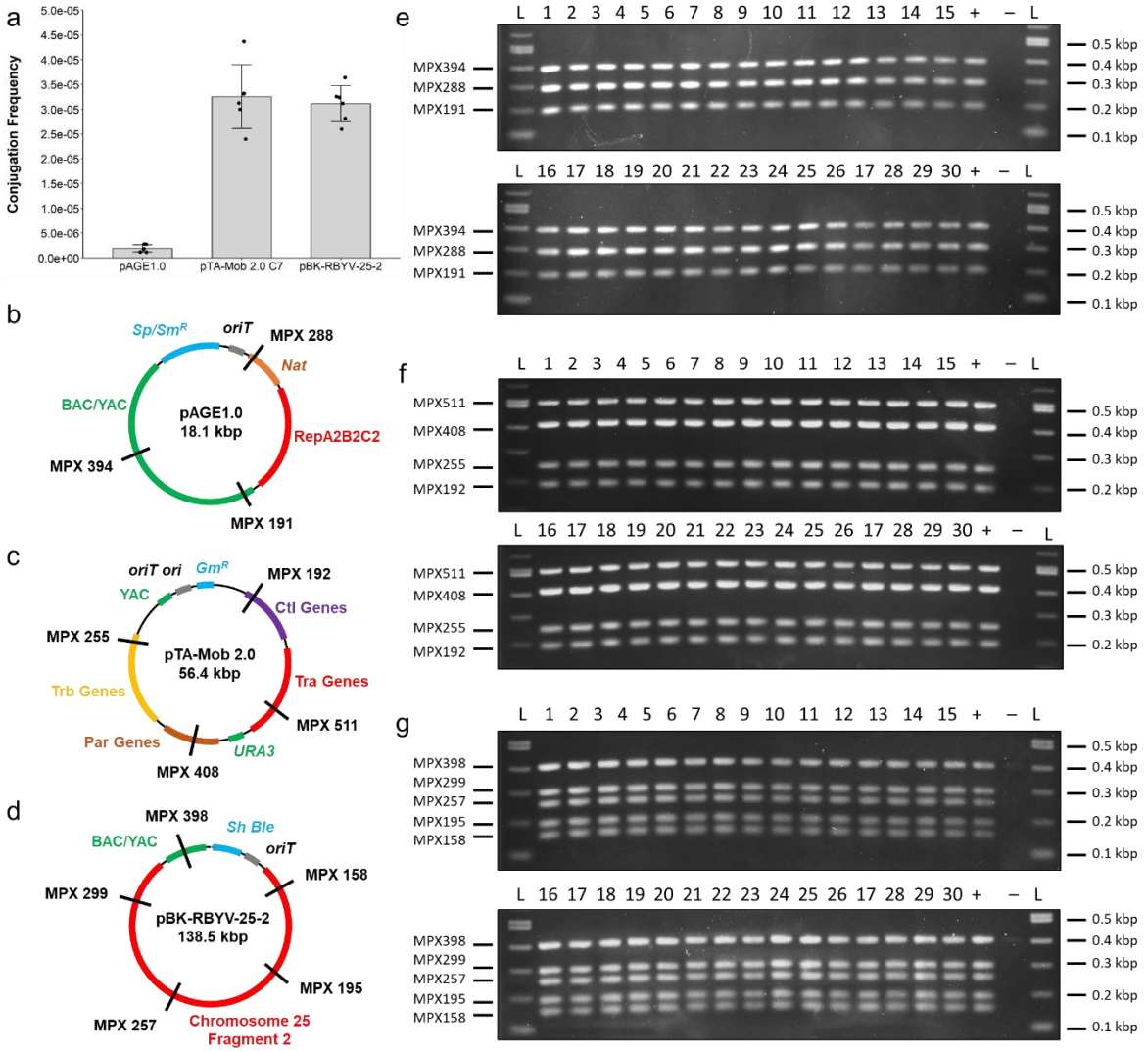


**Figure 4.** Conjugal transfer of plasmids increasing in size: (**a**) Conjugation frequency with donor *E. coli* containing either pTA-Mob 2.0 or pTA-Mob with either pAGE1.0 or pBK-RBYV-25-2 to recipient *S. cerevisiae*; Plasmid map and Multiplex PCR primer locations for (**b**) pAGE1.0; (**c**) pTA-Mob 2.0; or (**d**) pBK-RBYV-25-2; Diagnostic Multiplex PCR for 30 transconjugant *S. cerevisiae* colonies containing (**e**) pAGE1.0; (**f**) pTA-Mob 2.0; or (**g**) pBK-RBYV-25-2. Note: A faint band can be seen around 400 bp in the negative control of the Multiplex PCR for pBK-RBYV-25-2, which is most likely non-specific amplification.

To verify that entire plasmids were successfully transferred to yeast, we consecutively restreaked the yeast colonies three times on selective media containing ampicillin to remove any leftover donor *E. coli*. After the second passage, a portion of each colony was streaked onto an LB plate containing gentamicin and incubated at 37°C overnight. The LB plate did not yield any *E. coli* colonies after incubation, confirming that any leftover donor *E. coli* was eliminated during the passage of the yeast colonies. Plasmid DNA from the yeast transconjugants was then isolated after the third passage by lysing the cells in TE buffer and used for diagnostic Multiplex PCR (Figure 4e, f, g). For each plasmid, we tested 30 individual recipient colonies, and all 90 colonies yielded bands of the expected sizes. The Multiplex PCR results indicate that complete conjugal transfer of each plasmid was achieved for every colony tested and suggests that the frequency of conjugation to *S. cerevisiae*—when using either the pTA-Mob (*trans*) or pTA-Mob 2.0 (*cis*) system—does not decrease as plasmid size increases within the tested range using the solid media conjugation protocol.

## 4. Discussion

The development of engineered conjugative plasmids such as pTA-Mob [31], pLS20 [36], pRK2013 [18], pRH210 [37], and RP4 [38] provide a simple alternative for DNA transfer to prokaryotic and eukaryotic recipient cells. As recently demonstrated, the development of conjugation methods for species such as the eukaryotic algae *P. tricornutum* and other species allow for rapid advances in genetic engineering of these organisms [9,11,12]. In this study, we aimed to develop a pipeline for building conjugative plasmids and subsequently demonstrate that such plasmids can be efficiently delivered from a bacterial donor to a eukaryotic recipient within solid media. To achieve this we used *E. coli* as a conjugative donor and the model yeast *S. cerevisiae* as the eukaryotic recipient. Trans-kingdom conjugation to *S. cerevisiae* from *E. coli* had already demonstrated in liquid conditions and on the surface of solid media, providing a basis for the creation of the conjugation in solid media protocol [7,8].

First, we developed a PCR-based plasmid synthesis pipeline for building conjugative plasmids that can propagate in *E. coli* and yeast. Using this method, we found that 23 out of 30 plasmid clones tested were able to conjugate at least as well as the parental pTA-Mob 2.0 clone. After sequencing three of these plasmids, an average of 6.33 mutations per 56.4 kbp plasmid was found. It has been previously demonstrated that Takara PrimeSTAR GXL polymerase has an error rate of about 8.4 x $10^{-6}$ substitutions per base per PCR doubling [39]. If we consider each PCR cycle in the optimized protocol as a doubling, the error rate found in our plasmids was determined to be lower than the previously determined value, at around 4.5 x $10^{-6}$ mutations per base per PCR doubling, corresponding to a mutation about every 8.9 kbp. Some of these mutations may have arisen outside of the PCR conditions during plasmid assembly in yeast and or during propagation of plasmids in yeast or *E. coli*. If desired, mutations could be eliminated by first cloning each correct fragment flanked by preferred unique restriction sites in the plasmid of interest. Fragments could then be easily released by restriction digest, followed by yeast assembly. Since this pipeline utilizes PCR fragments for assembly, incorporating modular components and customizing vectors is rapid and simple. Additional components can be incorporated into the assembly mixture if there is built-in homology on the terminal ends of the fragment. Homology can be easily introduced during amplification by designing primers that contain a "hook" with sequence complementarity to the flanking DNA fragments. To delete components, such as an undesirable gene, a single fragment can be amplified as two separate fragments flanking the unwanted DNA region, creating a seamless deletion of the target. Not only can the pipeline generate functional conjugative plasmids—as indicated with the creation and testing of pTA-Mob 2.0—the pipeline can be adapted for use with any desired customizable vector.

During the optimization process, it was identified that the ideal holding temperature for the molten agar media, and at the time the cell mixture was added, was 60°C. Previous studies using different bacterial species have also demonstrated that a "heat shock" step during conjugation results in improved conjugation efficiencies [40–42]. However, it remains unclear as to why the heat shock at 60°C yields an increased number of transconjugant *S. cerevisiae* colonies. Next, we demonstrated that the number of transconjugant colonies per plate increased with higher agar composition, producing the best results with 2% agar prior to the addition of the cell mixture. As agar concentration increases, the movement of cells is restricted and, therefore, may provide additional stability of the pili during the formation of cell-to-cell attachments between bacteria and yeast. A molten media composition of higher than 2% agar was difficult to pour and impacted the ability to ensure the top layer was even for the conjugation plates. If needed, higher percentage compositions of the media could be achieved through the use of low-melting point agarose, to avoid premature solidification of the top layer.

Next, we demonstrated that cell density had a significant effect on yeast colony formation. After optimization, the most consistent conditions for transconjugant colony yields was achieved when

both donor and recipient cultures were harvested at an $OD_{600}$ of 1.0, and 100 µL of each cell resuspension was used (approximately 2.0 x $10^7$ yeast cells per mL of top agar based on colony-forming unit counts). For experiments where faster growth of yeast colonies is beneficial or to obtain larger transconjugant yeast colonies, decreasing the volume of cell resuspensions used can yield such a result. During the early optimization process, it was found that the thickness in the top agar layer had an impact on the formation of transconjugant yeast colonies (data not shown). Thus, it is essential that the total top agar plus cell mixture volume is 6 mL.

Once the protocol for conjugation within solid media was optimized, three plasmids of increasing size were then tested: pAGE1.0 (18.1 Kbp), pTA-Mob 2.0 (56.4 Kbp), and pBK-RBYV-25-2 (138.5 Kbp). For donor *E. coli* containing pAGE1.0 and pBK-RBYV-25-2, the vectors were mobilized using pTA-Mob as a helper plasmid for *trans* conjugation. In comparison, pTA-Mob 2.0 is able to self-transfer via *cis* conjugation. As both types of vectors are no longer mobilizable once in *S. cerevisiae*, the difference in colony yields would not be confounded by re-conjugation of recipients in the *cis* setup. It has been previously stated that conjugation efficiency decreases as vector size increases [5,43]. In this study, however, there was found to be no noticeable drop when comparing the transconjugant yields between the 56.4 kbp vector (pTA-Mob 2.0) and the 138.5 kbp vector (pBK-RBYV-25-2). From the Multiplex PCR reactions, we were able to confirm that the complete transfer of all three vectors occurred in every colony screened. With no substantial decrease in conjugation frequency and complete transfer of each vector confirmed, the upper size limit of conjugal transfer to eukaryotic cells has not yet been met. As for why pAGE1.0, a relatively small vector, did not conjugate as well as the larger plasmids, additional investigation is required.

Prior to this study, conjugation within solid media to yeast had not been demonstrated. This method allows for the transfer plasmids of at least 138.5 kbp; however, the upper size limit of conjugal transfer to eukaryotes has still yet to be determined. The protocol may be adjusted for transfer to other prokaryotic or eukaryotic organisms as well. For example, some culturing methods for microanaerobic and anaerobic bacteria rely on coating or mixing cells with a semi-solid agar media layer for growth and certain eukaryotic algae have been cultured on plates when allowed to grow within the agar layer of the plate itself [27–29]. Species that require semi-solid or solid media to grow within may be able to be engineered using an adapted version of this conjugation protocol. Additionally, protocols for more accurate enumeration of colony-forming units rely on submerging cells in agar and allowing an increased number of smaller colonies to grow and be counted per plate [30]. By eliminating the need for plating with a spreader, a more accurate count of cells in a sample can be made by removing the error arising from cells sticking to the spreader. Thus, with this optimized conjugation protocol, a more accurate enumeration of transconjugants can be estimated for experimental conditions. The result is a simple, accurate protocol for conjugal transfer to *S. cerevisiae* that permits the transfer of large vectors of at least up to 138.5 kbp.

**Supplementary Materials:** Supplementary materials are available online.

**Author Contributions:** Conceptualization, **M.P.M.S., B.J.K;** methodology, **M.P.M.S., R.S.M., S.H., P.J., B.J.K;** validation, **M.P.M.S., R.S.M., S.H., P.J;** investigation, **M.P.M.S., R.S.M., S.H., P.J., B.J.K;** formal analysis, **M.P.M.S.;** resources, **D.R.E., B.J.K;** writing—original draft preparation, **M.P.M.S., B.J.K;** writing—review and editing, **M.P.M.S., R.S.M., S.H., P.J., D.R.E., B.J.K;** visualization, **M.P.M.S., R.S.M., S.H., P.J., D.R.E., B.J.K;** supervision, **P.J., B.J.K;** project administration, **B.J.K;** funding acquisition, **B.J.K.**

**Funding:** This research was funded by: Defense Advanced Research Projects Agency (DARPA), Agreement Number: D18AC00035. The views, opinions and/or findings expressed are those of the author and should not be interpreted as representing the official views or policies of the Department of Defense or the U.S. Government.

Research in the labs of D.R.E., and B.J.K. is also supported by Natural Sciences and Engineering Research Council of Canada (NSERC), RGPIN-2015-04800, RGPIN-2018-06172 respectively. In addition, this study was supported by Designer Microbes Inc (DMI).

**Conflicts of Interest:** B.J.K. is Chief Executive Officer of Designer Microbes Inc. B.J.K and P.J. hold Designer Microbes Inc. stock.

## References


1. Cabezón, E.; Ripoll-Rozada, J.; Peña, A.; de la Cruz, F.; Arechaga, I. Towards an integrated model of bacterial conjugation. *FEMS Microbiol. Rev.* **2014**, *39*, 81–95.

2. Chilton, M.-D.; Drummond, M.H.; Merlo, D.J.; Sciaky, D.; Montoya, A.L.; Gordon, M.P.; Nester, E.W. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* **1977**, *11*, 263–271.

3. Christie, P.J. Agrobacterium tumefaciens T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria. *J. Bacteriol.* **1997**, *179*, 3085–3094.

4. Stachel, S.E.; Zambryski, P.C. Generic trans-kingdom sex? *Nature* **1989**, *340*, 190–191.

5. Heinemann, J.A.; Sprague, G.F. Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. *Nature* **1989**, *340*, 205–209.

6. Bundock, P.; den Dulk-Ras, A.; Beijersbergen, A.; Hooykaas, P.J. Trans-kingdom T-DNA transfer from Agrobacterium tumefaciens to Saccharomyces cerevisiae. *EMBO J.* **1995**, *14*, 3206–3214.

7. Moriguchi, K.; Yamamoto, S.; Ohmine, Y.; Suzuki, K. A Fast and Practical Yeast Transformation Method Mediated by Escherichia coli Based on a Trans-Kingdom Conjugal Transfer System: Just Mix Two Cultures and Wait One Hour. *PLoS One* **2016**, *11*, e0148989.

8. Brumwell, S.L.; MacLeod, M.R.; Huang, T.; Cochrane, R.R.; Meaney, R.S.; Zamani, M.; Matysiakiewicz, O.; Dan, K.N.; Janakirama, P.; Edgell, D.R.; et al. Designer Sinorhizobium meliloti strains and multi-functional vectors enable direct inter-kingdom DNA transfer. *PLoS One* **2019**, *14*, e0206781.

9. Karas, B.J.; Diner, R.E.; Lefebvre, S.C.; McQuaid, J.; Phillips, A.P.R.; Noddings, C.M.; Brunson, J.K.; Valas, R.E.; Deerinck, T.J.; Jablanovic, J.; et al. Designer diatom episomes delivered by bacterial conjugation. *Nat. Commun.* **2015**, *6*, 6925.

10. Diner, R.E.; Bielinski, V.A.; Dupont, C.L.; Allen, A.E.; Weyman, P.D. Refinement of the Diatom Episome Maintenance Sequence and Improvement of Conjugation-Based DNA Delivery Methods. *Front. Bioeng. Biotechnol.* **2016**, *4*, 65.

11. Slattery, S.S.; Diamond, A.; Wang, H.; Therrien, J.A.; Lant, J.T.; Jazey, T.; Lee, K.; Klassen, Z.; Desgagné-Penix, I.; Karas, B.J.; et al. An Expanded Plasmid-Based Genetic Toolbox Enables Cas9 Genome Editing and Stable Maintenance of Synthetic Pathways in Phaeodactylum tricornutum. *ACS Synth. Biol.* **2018**, *7*, 328–338.

12. Wang, H.; Slattery, S.; Karas, B.; Edgell, D. Delivery of the Cas9 or TevCas9 System into Phaeodactylum tricornutum via Conjugation of Plasmids from a Bacterial Donor. *BIO-*

*PROTOCOL* **2018**, *8*.

13. Waters, V.L. Conjugation between bacterial and mammalian cells. *Nat. Genet.* **2001**, *29*, 375–376.

14. Kunik, T.; Tzfira, T.; Kapulnik, Y.; Gafni, Y.; Dingwall, C.; Citovsky, V. Genetic transformation of HeLa cells by Agrobacterium. *Proc. Natl. Acad. Sci.* **2001**, *98*, 1871–1876.

15. Fernández-González, E.; de Paz, H.D.; Alperi, A.; Agúndez, L.; Faustmann, M.; Sangari, F.J.; Dehio, C.; Llosa, M. Transfer of R388 derivatives by a pathogenesis-associated type IV secretion system into both bacteria and human cells. *J. Bacteriol.* **2011**, *193*, 6257–6265.

16. Schröder, G.; Schuelein, R.; Quebatte, M.; Dehio, C. Conjugative DNA transfer into human cells by the VirB/VirD4 type IV secretion system of the bacterial pathogen Bartonella henselae. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 14643–14648.

17. Aune, T.E.V.; Aachmann, F.L. Methodologies to increase the transformation efficiencies and the range of bacteria that can be transformed. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 1301–1313.

18. Heinze, S.; Kornberger, P.; Grätz, C.; Schwarz, W.H.; Zverlov, V. V.; Liebl, W. Transmating: conjugative transfer of a new broad host range expression vector to various Bacillus species using a single protocol. *BMC Microbiol.* **2018**, *18*, 56.

19. Karas, B.J.; Jablanovic, J.; Sun, L.; Ma, L.; Goldgof, G.M.; Stam, J.; Ramon, A.; Manary, M.J.; Winzeler, E.A.; Venter, J.C.; et al. Direct transfer of whole genomes from bacteria to yeast. *Nat. Methods* **2013**, *10*, 410–412.

20. Hill, K.E.; M. Top, E. Gene transfer in soil systems using microcosms. *FEMS Microbiol. Ecol.* **1998**, *25*, 319–329.

21. Brophy, J.A.N.; Triassi, A.J.; Adams, B.L.; Renberg, R.L.; Stratis-Cullum, D.N.; Grossman, A.D.; Voigt, C.A. Engineered integrative and conjugative elements for efficient and inducible DNA transfer to undomesticated bacteria. *Nat. Microbiol.* **2018**, *3*, 1043–1053.

22. Ronda, C.; Chen, S.P.; Cabral, V.; Yaung, S.J.; Wang, H.H. Metagenomic engineering of the mammalian gut microbiome in situ. *Nat. Methods* **2019**, *16*, 167–170.

23. Itaya, M.; Sato, M.; Hasegawa, M.; Kono, N.; Tomita, M.; Kaneko, S. Far rapid synthesis of giant DNA in the Bacillus subtilis genome by a conjugation transfer system. *Sci. Rep.* **2018**, *8*, 8792.

24. Zechner, E.L.; Moncalián, G.; de la Cruz, F. Relaxases and Plasmid Transfer in Gram-Negative Bacteria. In *Type IV Secretion in Gram-Negative and Gram-Positive Bacteria*; Backert, S., Grohmann, E., Eds.; Springer, Cham, 2018; Vol. 413, pp. 93–113 ISBN 978-3-319-75241-9.

25. Christie, P.J. Type IV secretion: Intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. *Mol. Microbiol.* 2001, *40*, 294–305.

26. Bradley, D.E.; Taylor, D.E.; Cohen, D.R. Specification of surface mating systems among conjugative drug resistance plasmids in Escherichia coli K-12. *J. Bacteriol.* **1980**, *143*, 1466–1470.

27. Mason, J.H. Isolation of anaerobic bacteria by a modified shake method. *J. Gen. Microbiol.* **1953**, *8*, 263–264.

28. Nagasaki, K.; Imai, I. Solid-phase culture of marine phytoflagellates. *Bull. Japanese Soc. Mircobial Ecol.* **1994**, *9*, 37–43.

29. Lakeman, M.B.; Cattolico, and R.A. Cryptic Diversity in Phytoplankton Cultures is Revealed Using a Simple Plating Technique. *J. Phycol.* **2007**, *43*, 662–674.

30. Koch, A.L. Growth Measurement. In *Methods for General and Molecular Biology*; Gerhard, P., Murray, R.G.E., Wood, W.A., Krieg, N.R., Eds.; American Society for Microbiology Press: Washington, D.C., 2007; pp. 172–199.

31. Strand, T.A.; Lale, R.; Degnes, K.F.; Lando, M.; Valla, S. A new and improved host-independent plasmid system for RK2-based conjugal transfer. *PLoS One* **2014**, *9*, e90372.

32. Karas, B.J.; Jablanovic, J.; Irvine, E.; Sun, L.; Ma, L.; Weyman, P.D.; Gibson, D.G.; Glass, J.I.; Venter, J.C.; Hutchison, C.A.; et al. Transferring whole genomes from bacteria to yeast spheroplasts using entire bacterial cells to reduce DNA shearing. *Nat. Protoc.* **2014**, *9*, 743–750.

33. Hanahan, D. Studies on transformation of Escherichia coli with plasmids. *J. Mol. Biol.* **1983**, *166*, 557–580.

34. Diner, R.E.; Noddings, C.M.; Lian, N.C.; Kang, A.K.; McQuaid, J.B.; Jablanovic, J.; Espinoza, J.L.; Nguyen, N.A.; Anzelmatti, M.A.; Jansson, J.; et al. Diatom centromeres suggest a mechanism for nuclear DNA acquisition. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, E6015–E6024.

35. Karas, B.J.; Moreau, N.G.; Deerinck, T.J.; Gibson, D.G.; Venter, J.C.; Smith, H.O.; Glass, J.I. Direct Transfer of a Mycoplasma mycoides Genome to Yeast Is Enhanced by Removal of the Mycoides Glycerol Uptake Factor Gene glpF. *ACS Synth. Biol.* **2019**, *8*, 239–244.

36. Miyano, M.; Tanaka, K.; Ishikawa, S.; Takenaka, S.; Miguel-Arribas, A.; Meijer, W.J.J.; Yoshida, K. ichi Rapid conjugative mobilization of a 100kb segment of Bacillus subtilis chromosomal DNA is mediated by a helper plasmid with no ability for self-transfer. *Microb. Cell Fact.* **2018**, *17*, 13.

37. Moriguchi, K.; Yamamoto, S.; Tanaka, K.; Kurata, N.; Suzuki, K. Trans-Kingdom Horizontal DNA Transfer from Bacteria to Yeast Is Highly Plastic Due to Natural Polymorphisms in Auxiliary Nonessential Recipient Genes. *PLoS One* **2013**, *8*, e74590.

38. Quandt, J.; Clark, R.G.; Venter, A.P.; Clark, S.R..; Twelker, S.; Hynes, M.F. Modified RP4 and Tn5-Mob derivatives for facilitated manipulation of large plasmids in Gram-negative bacteria. *Plasmid* **2004**, *52*, 1–12.

39. Potapov, V.; Ong, J.L. Examining Sources of Error in PCR by Single-Molecule Sequencing. *PLoS One* **2017**, *12*, e0169774.

40. Zhang, S.; Chen, T.; Jia, J.; Guo, L.; Zhang, H.; Li, C.; Qiao, R. Establishment of a highly efficient conjugation protocol for Streptomyces kanamyceticus ATCC12853. *Microbiologyopen* **2019**, *8*, e00747.

41. Zeng, X.; Ardeshna, D.; Lin, J. Heat Shock-Enhanced Conjugation Efficiency in Standard Campylobacter jejuni Strains. *Appl. Environ. Microbiol.* **2015**, *81*, 4546–4552.

42. Kirk, J.A.; Fagan, R.P. Heat shock increases conjugation efficiency in Clostridium difficile. *Anaerobe* **2016**, *42*, 1–5.

43. Heinemann, J.A.; Sprague, G.F. Transmission of Plasmid DNA to Yeast by Conjugation with Bacteria. *Methods Enzymol.* **1991**, *194*, 187–195.