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Conjugative Mating Assays for Sequence-Specific Analysis of Transfer Proteins Involved in Bacterial Conjugation

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Abstract:	The transfer of genetic material by bacterial conjugation is a process that takes place via complexes formed by specific transfer proteins. In Escherichia coli, these transfer proteins make up a DNA transfer machinery known as the mating pair formation, or DNA transfer complex, which facilitates conjugative plasmid transfer. The objective of this paper is to provide a method that can be used to determine the role of a specific transfer protein that is involved in conjugation using a series of deletions and/or point mutations in combination with mating assays. The target gene is knocked out on the conjugative plasmid and is then provided in trans through the use of a small recovery plasmid harboring the target gene. Mutations affecting the target gene on the recovery plasmid can reveal information about functional aspects of the target protein that result in the alteration of mating efficiency of donor cells harboring the mutated gene. Alterations in mating efficiency provide insight into the role and importance of the particular transfer protein, or a region therein, in facilitating conjugative DNA transfer. Coupling this mating assay with detailed three-dimensional structural studies will provide a comprehensive understanding of the function of the conjugative transfer protein as well as provide a means for identifying and characterizing regions of protein-protein interaction.
Author Comments:	Thank you for your editorial review and revisions. We have addressed the concerns noted and revised the manuscript accordingly. In particular, we have revised the text

	substantially, rewording the Introduction, Representative Results and Discussion sections substantially, provided the protocol in the imperative case, rechecked all spelling/grammar, double checked numbering within the protocol etc., provided DOIs for references and highlighted in yellow the important steps. We have also revised figures and tables to be more reflective and streamlined for the publication.
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TITLE:
Conjugative Mating Assays for Sequence-Specific Analysis of Transfer Proteins Involved in Bacterial Conjugation

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KEYWORDS:
Mating assay; mating efficiency; *Escherichia coli*; bacterial conjugation; F plasmid; type 4 secretion system; donor cell; recipient cell

SHORT ABSTRACT:
Here, we present a protocol to knockout a gene of interest involved in plasmid conjugation and subsequently analyze the impact of its absence using mating assays. The function of the gene is further explored to a specific region of its sequence using deletion or point mutations.

LONG ABSTRACT:
The transfer of genetic material by bacterial conjugation is a process that takes place via complexes formed by specific transfer proteins. In *Escherichia coli*, these transfer proteins make up a DNA transfer machinery known as the mating pair formation, or DNA transfer complex, which facilitates conjugative plasmid transfer. The objective of this paper is to provide a method that can be used to determine the role of a specific transfer protein that is involved in conjugation using a series of deletions and/or point mutations in combination with mating assays. The target gene is knocked out on the conjugative plasmid and is then provided *in trans* through the use of a small recovery plasmid harboring the target gene. Mutations affecting the target gene on the recovery plasmid can reveal information about functional aspects of the target protein that result in the alteration of mating efficiency of donor cells harboring the mutated gene. Alterations in mating efficiency provide insight into the role and importance of the particular transfer protein, or a region therein, in facilitating conjugative DNA transfer. Coupling this mating assay with detailed three-dimensional structural studies will provide a comprehensive understanding of the function of the conjugative transfer protein as well as provide a means for identifying and characterizing regions of protein-protein interaction.

INTRODUCTION:

The transfer of genes and proteins at the micro-organismal level plays a central role in bacterial survival and evolution as well as infection processes. The exchange of DNA between bacteria or between a bacterium and a cell can be achieved through transformation, conjugation or vector transduction.[1,2] Conjugation is unique in comparison to transformation and transduction in that during conjugation between gram-negative bacteria such as *Escherichia coli*, the transfer of DNA occurs in a donor-controlled fashion whereby a complex macromolecular system connects donor and recipient cells. Conjugation is also the most direct way in which bacterial cells interact with host cells to inject genes, proteins or chemicals in to host systems.[3] Quite often, the transfer of such agents has remarkable effects on the host, ranging from pathogenesis and carcinogenesis to host evolution and adaptation. It has been shown that conjugative recombination increases the rate of adaptation 3-fold in bacteria with high mutation rates under conditions of environmental stress.[4] Moreover, conjugation is by far the most common route through which antibiotic resistance genes in bacterial strains are spread.[5,6]

Microorganisms have evolved specialized secretion systems to support the transfer of macromolecules across cellular membranes; there are currently 9 types of secretion systems

(TSSs) in gram negative bacteria that have been described: T1SS, T2SS, T3SS, T4SS, T5SS, T6SS, T7SS, as well as the Sec (secretion) and Tat (two-arginine translocation) pathways.[7,8] Each type of secretion system is further divided into different subtypes, a necessity due to diversity of proteins and the distinctiveness of pathways involved, in different bacterial strains. For example, in the type IV secretion system (T4SS), the Ti and Cag systems facilitate effector transport whereas the F-plasmid, R27 and pKM101 T4SSs facilitate transfer of a conjugative plasmid.[7,9,10] A detailed understanding of the mechanisms by which organisms assemble their respective secretion systems from their component proteins and share cellular contents with a recipient or their surrounding environment is an important factor in development of targeted strategies to combat pathogenic microorganisms and processes of cellular infection.

Following the initial identification bacterial conjugation in *E. coli* by Lederberg & Tatum,[11] a large number of mobile and conjugative plasmids have been identified and characterized.[12] Such mobile plasmids show considerable range is size (from 1 to over 200 kilobases (kb)), however all mobile plasmids contain a relaxase, which recognizes the origin of transfer (oriT) thereby enabling transmission of the plasmid. Conjugative plasmids further encode genes for assembly of a functional T4SS as well as a type IV coupling protein.[12] For example the 100 kb F plasmid of *E. coli* encodes all the conjugative genes within a 33.3 kB transfer (tra) region.[13] The genes in the *tra* region of the F plasmid encode all proteins that facilitate pilus formation, mating pair formation (Mpf), DNA transfer and exclusion functions during conjugative plasmid transfer.[10,14,15] A significant body of knowledge is available for conjugative T4SSs, however detailed structural studies of the conjugative proteins and complexes are only more recently becoming available.[16–28]

In order to assemble a comprehensive view of the conjugative process, a coupling of detailed structural studies to mutational analyses of conjugative transfer proteins is required. This can be achieved through conjugative mating assays. For the F plasmid, each protein encoded within the *tra* region plays a role in the F-mediated conjugation; therefore, the knockout/deletion of a transfer gene will abolish the conjugative capacity of the cell (**Figure 1**). While smaller mobile plasmids are more conducive to standard deletion procedures, for larger conjugative plasmids such as F, gene knockouts are more readily achieved via homologous recombination where the target gene is replaced with one conveying a distinct antibiotic resistance gene. In the current protocol, we employ homologous recombination to replace a transfer gene of interest with chloramphenicol acetyltransferase (CAT) in the 55 kb F plasmid derivative pOX38-Tc;[29,30] the resultant knockout plasmid, pOX38-Tc Δgene::Cm, facilitates resistance to the presence of chloramphenicol (Cm) in the growth media. Donor cells harboring pOX38-Tc Δgene::Cm are unable to affect conjugative DNA transfer/mating as observed through the use of a mating assay; the mating efficiency of a pOX38-Tc Δgene::Cm donor cell and a normal recipient will decrease or, more often, be abolished. Conjugative transfer of the pOX38-Tc Δgene::Cm plasmid can be restored via a small recovery plasmid harbouring the targeted transfer gene. This recovery plasmid can be one that provides constitutive expression, such as plasmid pK184 (pK184-gene),[31] or one that provides inducible expression so long as that plasmid properly targets the gene to the correct location within the cell (cytoplasm or periplasm). Consequently, in mating assays between this new donor (harbouring pOX38-Tc Δgene::Cm + pK184-gene plasmids) and a recipient cell, the mating efficiency is expected to restore to nearly that of a normal donor-recipient mating assay. This system enables one to probe the function of the knocked out gene

through the generation of a series of pK184-gene constructs (deletions or point mutations) and testing each construct's ability to restore the mating capacity of the pOX38-Tc Δgene::Cm harboring donor cells.

PROTOCOL:

1. Generation of DNA constructs

1.1. Designing Oligomers for homologous recombination of the target gene

1.1.1. Design a single 55-72 bp forward oligomer as follows: (a) pick a 19-32 bp long nucleotide sequence that is homologous to a DNA sequence in the region 10-100 bp upstream of the 5' start site of the chloramphenicol acetyltransferase gene in the commercial pBAD33 plasmid,[32] and (b) select a 36-54 bp long nucleotide sequence homologous to a region 10-150 bp downstream of the 5' start site of the target gene of interest.

1.1.1.1. Join the 3' end of nucleotide sequence picked in (b) to the 5' end of the nucleotide sequence picked in (a), thus giving a single 55-72 long forward oligomer.

1.1.2. Design a single 55-72 bp reverse oligomer as follows: (a) pick a 19-32 bp long nucleotide sequence that is homologous to a DNA sequence in the region 10-100 bp downstream of the 3' end of the chloramphenicol gene in the commercial pBAD33 plasmid, and (b) select a 36-54 bp long nucleotide sequence homologous to a region within 10-150 bp upstream of the 3' end site of the target gene of interest.

1.1.2.1. Join the 3' end of nucleotide sequence picked in (b) to the 5' end of the nucleotide sequence picked in (a) to make it a single oligomer.

1.1.2.2. Copy this oligomer into any available bioinformatics software and convert sequence into its reverse complement. This will give a single 55-72 bp long reverse oligomer.

1.1.3. Using any available oligo-analyzer program, check that the recombination oligomers have GC content between 40-60%, low hairpin melting temperature (T_m), low self- and hetero-dimerization T_m's. Order the primers for synthesis and shipment from any preferred biotechnology company.

1.2. Amplification of the CAT cassette from pBAD33 (Cm^R) plasmid using oligomers designed for homologous pairing

1.2.1. Grow an overnight (O/N, 16-18 h) culture of DH5α cells harboring the pBAD33 plasmid in sterile Lysogeny broth (LB) with 20 µg/mL chloramphenicol (Cm) with shaking at 200 rpm and 37 °C.

1.2.2. Centrifuge 6-8 mL of the O/N culture at room temperature, 5000 x g for 3 min. Decant the supernatant and extract the pBAD33 plasmid from the pellet using a plasmid mini-prep kit (Materials Table) and manufacturer's protocol.

1.2.3. Do a digest of the pBAD33 plasmid DNA by adding the following into a sterile tube in the order given: appropriate volume of double distilled water (ddH_2O) to a final volume of 50

µL, 1 µg volume of pBAD33 plasmid, 5 µL 10X enzyme reaction buffer, and 0.5 µL of AvaI restriction enzyme (RE). Gently mix by pipetting and let the reaction proceed for 1 h at 37 °C. Heat inactivate the reaction for 20 minutes (min) at 80 °C. Store samples at -20 °C for no longer than 24 h to minimize sticky-end degradation.

1.2.4. Prepare a 1.2% agarose separating gel by mixing 1.2 g of agarose with 100 mL of 1X TAE (40 mM Tris, pH 8.5; 20 mM acetic acid; 1 mM EDTA) buffer in a 250 mL Erlenmeyer flask. Heat and swirl to completely dissolve in a microwave. Stop heating immediately when the liquid begins to boil and swirl the flask.

1.2.4.1. Cool the liquid agarose for 3 min at room temperature while swirling, add 2 µL of 10 mg/mL ethidium bromide and swirl to mix. Pour the agarose into a gel tray with a well comb and allow it to solidify for 1 h at room temperature. Store gels at 4 °C for up to 2 days in 1X TAE buffer.

1.2.5. Mix each RE digest from 1.2.3 with 0.2 volume of DNA loading dye (10 µL of dye per 50 µL reaction) by pipetting. Load 5 µL of 500 µg/mL DNA ladder into the first well and all of the RE digest-dye mixture into another well on the agarose gel. Use 2-3 wells to load all of the reaction volume onto the gel.

1.2.5.1. Run the gel barely submerged in 1X TAE buffer and operating at 45-50 V (4.5-5 V/cm) for 65 min in a gel electrophoresis device.

1.2.6. Using a UV cabinet and a sterile razor, quickly cut the 2.8 kb band that corresponds to the CAT sequence out of the gel to minimize UV exposure of DNA. Extract the DNA from the cut-out gel slice using a gel extraction kit (Materials Table) as per the manufacturer's protocol.

Note: Take care not to expose skin directly under UV and handle the razor with care.

1.2.7. Amplify the CAT cassette extracted from 1.2.6 by Polymerase Chain Reaction (PCR) using the primers designed in 1.1 that contain overhangs homologous to the gene sequence to allow for homologous recombination. PCR reactions are set up on ice using the manufacturer guidelines (Materials Table) in the following order:

1.2.7.1. Into a sterile PCR tube, add an appropriate volume of ddH_2O to a final volume of 50 µL, followed by 10 µL of PCR buffer, 1 µL of 10 mM dNTPs, 2.5 µL of 10 µM Forward primer, 2.5 µL of 10 µM Reverse primer, 1-25 ng of template DNA from 1.2.6 and 0.5 µL of 100 units/µL DNA polymerase.

1.2.7.2. Set up a negative control that bears the same components as 1.2.7.1 but with the exception of template DNA. Use an appropriate volume of ddH_2O instead of template DNA. Set up a positive control using template DNA and primers that have been proven to work in a PCR reaction, such as those provided as positive control by the manufacturer.

1.2.7.3. Mix all reaction contents gently by pipetting.

1.2.7.4. Amplify via PCR using the following settings: Initial denaturation for 30 s at 98 ˚C, 30 cycles of denaturation for 10 s at 98 ˚C, primer annealing for 20 s, extension for 20 s per kilobase of amplicon at 72 ˚C and a final extension for 10 min at 72 ˚C. Store samples at -20 ºC.

1.2.8 Confirm the correct size of amplification via agarose gel electrophoresis (see 1.2.4-1.2.5) by using only 5 µL of each reaction. Purify the PCR amplicon using a PCR purification kit and manufacturer's protocol. Store purified DNA at -20 ºC.

1.3. The pK184-gene **recovery plasmid**

1.3.1. Design a forward primer beginning from its 5' end and going towards the 3' end in the following order: (a) pick 4 random nucleotides (a combination of adenine, thymine, guanine and cytosine) for cleavage efficiency, attached to (b) the EcoRI restriction enzyme (RE) cut site sequence (GAATTC), followed by (c) a 21-25 bp long nucleotide sequence that is homologous to the 5' end of the gene of interest, including the start codon. If the target gene contains an EcoRI site, choose another appropriate RE from the pK184 multiple cloning site.

1.3.2. Design a reverse primer in the following order: (a) pick 4 random nucleotides for cleavage efficiency, followed by (b) a HindIII cut site sequence (AAGCTT) followed by (c) the 21-25 bp long reverse complement of the 3' end of the gene of interest including the stop codon. If the target gene contains a HindIII site, choose another RE in the pK184 multiple cloning site.

1.3.3. In the case of genes encoding periplasmic proteins, include an additional leader sequence between the RE site and the start codon on the forward primer.

1.3.4. Using any available oligo-analyzer, check that the primers have GC content between 40-60%, low hairpin T_m, low self- and hetero-dimerization T_m's. Order the primers for synthesis.

1.3.5. Grow an O/N culture of DY330R pOX38-Tc cells in sterile LB containing 10 µg/mL tetracycline (Tc) with shaking at 32 ºC and 200 rpm. Centrifuge 6-8 mL of the O/N culture at room temperature, 5000 x g for 3 min. Decant the supernatant and extract the plasmid DNA from the pellet using a plasmid mini-prep kit (Materials Table) and manufacturer's protocol.

1.3.6. Amplify the full gene of interest with the primers from 1.3.1-1.3.5 using pOX38-Tc as the template (see 1.2.7.1-1.2.7.3).

1.3.7. Confirm the correct size of amplification via agarose gel electrophoresis (see 1.2.4-1.2.5) by using only 5 µL of each reaction. Purify the amplified DNA using a PCR purification kit and manufacturer's protocol. Store purified DNA at -20 ºC.

1.3.8. Do a double digest of both the commercially available pK184 plasmid DNA and the amplified gene (from 1.3.7.), by adding the following into a sterile tube in the order given: appropriate volume of ddH_2O to a final volume of 50 µL, 1 µg volume of pK184 plasmid, 5 µL 10X enzyme reaction buffer, and 1 µL of each EcoRI and HindIII.

1.3.8.1. Gently mix by pipetting and let the reaction proceed for 1 h at 37 ºC. Heat inactivate the reaction for 20 min at 80 ºC. Store samples at -20 °C for no longer than 24 h to minimize sticky-end degradation.

Note: The type of restriction nuclease used here depends on the restriction nuclease site that was engineered into the primers in steps 1.3.1 and 1.3.2.

1.3.8.2. As a positive control, set up single digests of the pK184 plasmid by preparing the same reaction as in 1.3.8 except add only one of the REs to a reaction tube. Do this separately with both REs.

1.3.9. Run the digests on a 1.2% agarose gel using protocols 1.2.4.-1.2.5. Extract the DNA double digest fragments of both pK184 and the gene of interest according to step 1.2.6.

1.3.10. Ligate the gene insert into the pK184 vector by adding components into a sterile tube in the following order: 2 µL of 10X T4 DNA Ligase Buffer, a total of 100 ng DNA composed of a 1:3 vector:insert (pK184:gene) ratio, ddH_2O up to a total volume of 20 µL and 1 µL T4 DNA ligase. As a negative control, set up a similar reaction using 100 ng of vector without the insert gene.

1.3.10.1. Gently mix all reaction contents using a micropipette and incubate for 30 min at 25 °C. Heat-inactivate the ligation reaction for 10 min at 65 °C and then place on ice.

1.3.11. While on ice, add 15 µL of the pK184-gene ligation reaction from step 1.3.10 to 100 µL of chemically competent DH5α cells in a sterile 1.5 mL tube. Gently mix by pipetting and incubate on ice for 10 minutes. Do the same for the negative control sample. For a positive control, use 20-100 ng of a plasmid such as pBAD33 and transform it into 50 µL of DHα cells.

1.3.12. Directly transfer the samples from ice into a 42 °C water bath and incubate for 90 seconds. This provides the cells a heat shock and allows them to uptake the plasmid DNA.

1.3.13. Place cells back on ice for another 5 minutes and then add 900 µL of sterile LB. Incubate at 37 °C for 1 h while shaking at 125 rpm.

1.3.14. Aliquot a 100 µL volume of sample from each ligation reaction in 1.3.13 onto an agar plate containing 50 µg/mL kanamycin (Km) and spread the cells using a sterile spreader. The positive control plate should have appropriate antibiotics. Keep the area sterile and work near a flame. Incubate the plate upside down at 37 °C overnight.

1.3.15. Using a sterile pipette or loop, harvest a single distinct colony of cells and inoculate a 20 mL sterile LB with 50 µg/mL Km. Keep the area sterile and work near a flame. Grow the cells O/N at 37 °C with shaking at 200 rpm.

1.3.16. Make 3-5 glycerol stocks of the transformed DH5α cells by mixing 500 µL of the O/N culture with 500 µL of sterile 100% glycerol (final 50% v/v) in sterile cryo-tubes. Store at -80 °C.

1.3.17. Also centrifuge 6-8 mL of the O/N culture at room temperature, 5000 x g for 3 min. Decant the supernatant and extract the pK184-gene recovery plasmid from the pellet using a plasmid mini-prep kit (Materials Table) and manufacturer's protocol.

1.4. pK184-gene Mutants

Note: Primers designed for deletions, insertions and/or point mutations can be easily generated using manufacturers' online available tools.

1.4.1. Design each forward primer by picking an 18-32 bp long nucleotide sequence that is homologous to the 5' end of the gene of interest, including the start codon. Design primers such that each forward primer anneals 30-180 bp downstream of the preceding one, resulting in deletion mutants lacking N-terminal peptide fragments of appropriate lengths.

1.4.2. Design a reverse primer by picking an 18-32 bp long nucleotide sequence that is homologous to the 3' end of the gene of interest including the stop codon. Copy this primer into any available bioinformatics program and convert the sequence into its reverse complement. This is the reverse primer.

1.4.2.1. In the case of genes encoding periplasmic proteins, design the reverse primer that is the reverse complement of the 3' end of a leader sequence that flanks the 5' end of the gene and is required for proper localization of the protein product in the periplasm.

1.4.3. Using any available oligo-analyzer program, check that the deletion primers have GC content between 40-60%, low hairpin melting temperature (T_m), low self- and hetero-dimerization T_m's. Order the primers for synthesis and shipment from any available biotechnology company.

1.4.4. Using the pK184-gene construct obtained in Protocol 1.3 as the template and the guidelines in 1.2.7-1.2.8, PCR amplify the deletion constructs with the primers designed in steps 1.4.1-1.4.3 to generate pK184-gene$_{\Delta X}$ amplicons. Store amplified DNA at -20 °C.

1.4.5. Ligate the amplified construct using any available mutagenesis kit (Materials Table).

1.4.6. Transform each of the pK184-gene$_{\Delta X}$ ligates separately into chemically competent DH5α cells using a standard heat shock protocol (see 1.3.11-1.3.13).

1.4.7. Aliquot a 100 µL volume of sample from each ligation reaction in 1.4.12 onto an agar plate containing 50 µg/mL Km and spread the cells using a sterile spreader. Keep the area sterile and work near a flame. Incubate the plate upside down at 37 °C overnight.

1.4.8. Using a sterile pipette or loop, harvest a single distinct colony of cells and inoculate a 20 mL sterile LB media with 50 µg/mL Km. Keep the area sterile and work near a flame. Grow the cells O/N at 37 °C with shaking at 200 rpm.

1.4.9. Make 3-5 glycerol stocks of the transformed DH5α cells by mixing 500 µL of the O/N culture with 500 µL of sterile 100% glycerol (final 50% v/v) in sterile cryo-tubes. Store at -80 °C.

1.4.10. Centrifuge 6-8 mL of the O/N culture at room temperature, 5000 x g for 3 min. Decant the supernatant and extract the pK184 gene$_{\Delta X}$ plasmid construct from the pellet using a plasmid mini-prep kit (Materials Table) and manufacturer's protocol. Store DNA at -20 °C.

2. Generation of pOX38-Tc Δgene::Cm strains

2.1. DY330R pOX38-Tc Δgene::Cm knockouts

2.1.1. Prepare an O/N culture of DY330R pOX38-Tc cells in 10 mL sterile LB containing 10 µg/ml Tc. Grow culture O/N at 32 °C and 200 rpm.

2.1.2. Make a 1:70 dilution of the O/N culture into 20 mL of fresh sterile LB. Grow the cells at 32 °C until mid-log phase (OD_{600nm} 0.4-0.6) growth.

2.1.3. Transfer 10 mL of culture to a sterile flask and incubate at 42 °C for 15 min at 150 rpm in a shaking water bath. This will induce the expression of recombination specific proteins in DY330R.

2.1.4. Chill the culture in an ice-water bath for 10 min. Prepare electrocompetent cells as follows.

2.1.4.1. Transfer the chilled cells into pre-chilled conical tubes and centrifuge at 4000 x g for 7 min at 4 ºC. All tubes and pipettes to be used in the upcoming steps should be placed at 4 °C or on ice to cool.

2.1.4.2. Remove the supernatant and gently resuspended the cells in 1 mL of ice-cold ddH_2O. Add another 30 mL of ice cold ddH_2O.

2.1.4.3. Centrifuge the cells (4000 x g, 7 min, 4 °C), discard the supernatant and gently resuspended the cells in 1 mL of ice-cold ddH_2O.

2.1.4.4. Transfer the resuspended cells into pre-chilled 1.5 mL microfuge tubes and centrifuge at 15000 x g for 1 min at 4 ºC.

2.1.4.5. Gently resuspended the pellet in 200 µL of ice-cold ddH_2O and aliquot in 50 µL volumes. Electrocompetent cells can be stored at -80 °C

2.1.5. Add 300 ng of the amplified CAT cassette from 1.2 into 50 µL of electrocompetent DY330R pOX38-Tc cells while mixing on ice, gently by pipetting up and down. Repeat this step using unmodified pBAD33 plasmid as a positive control.

2.1.6. Transfer the cells to a pre-cooled (-20 °C) 1 mm electroporation cuvette. Electroporate the cells at 1.8 kV with a time constant of 5.5 ms, using an electroporator. Immediately after applying the pulse, dilute the cells with 1 mL of SOC media and transfer to a fresh microfuge tube. Incubate the cells at 32 °C for 2 h.

2.1.7. Aliquot 100 µL of each sample onto agar plates containing 10 µg/mL Tc and 20 µg/mL Cm and spread using a sterile spreader. Keep the area sterile and work near a flame. Incubate the plate upside down at 32 °C overnight to select for the successful recombinants. The CAT cassette introduced into the cell will undergo homologous recombination with the gene of interest and create the DY330R pOX38-Tc Δgene::Cm (Rif^R, Tc^R, Cm^R) clone.

Note: It is important to grow DY330R cells at 32 °C, with the exception of the 15 min induction at 42 °C of Step 2.1.3 prior to generating electrocompetent cells, as prolonged growth at elevated temperatures risks cell death due to the production of toxic products from the p_L operon responsible for recombination functions in DY330R.[33,34]

2.1.8. Prepare an O/N of DY330R pOX38-Tc Δgene::Cm cells by harvesting a single distinct colony of cells with a sterile pipette or loop, and inoculating a 20 mL sterile LB media with 10 µg/mL Tc, and 20 µg/mL Cm. Keep the area sterile and work near a flame. Grow the cells O/N at 32 °C with shaking at 200 rpm.

2.1.9. Make 3-5 glycerol stocks from the O/N by mixing 500 µL of the O/N culture with 500 µL of sterile 100% glycerol (final 50% v/v) in sterile cryo-tubes. Store at -80 °C.

2.1.10. Centrifuge 6-8 mL of the O/N culture at room temperature, 5000 x g for 3 min. Decant the supernatant and extract the pOX38-Tc Δgene::Cm construct from the pellet using a plasmid mini-prep kit (Materials Table) and manufacturer's protocol; store purified DNA at -20 °C.

2.1.11. Perform a conjugative mating assay using XK1200 cells as the recipient to confirm disruption of conjugation by gene knockout as per protocol 3.1.

2.2. DY330R pOX38-Tc Δgene::Cm + pK184-gene

2.2.1. Transform electrocompetent DY330R pOX38-Tc Δgene::Cm cells with 300 ng of the pK184-gene construct via electroporation as per steps 2.1.4-2.1.7. All selective media must contain 20 µg/mL Cm and 50 µg/mL Km. Incubate at 32 °C. The recovery plasmid in the electroporated cells will now restore the function of the knocked out gene in the DY330R pOX38-Tc Δgene::Cm cells.

2.2.2. Prepare an O/N of DY330R pOX38-Tc Δgene::Cm + pK184-gene recombinant cells by harvesting a single distinct colony of the cells with a sterile pipette or loop, and inoculating a 20 mL sterile LB media with 20 µg/mL Cm and 50 µg/mL Km. Keep the area sterile and work near a flame. Grow the cells O/N at 32 °C with shaking at 200 rpm.

2.2.3. Make 3-5 glycerol stocks from the O/N by mixing 500 µL of the O/N culture with 500 µL of sterile 100% glycerol (final 50% v/v) in sterile cryo-tubes. Store at -80 °C.

2.2.4. Also perform protocol 3.1 to generate XK1200 pOX38-Tc Δgene::Cm recombinant cells.

2.3. XK1200 pOX38-Tc Δgene::Cm + pK184-gene and Mutants

2.3.1. Prepare electrocompetent XK1200 pOX38-Tc Δgene::Cm cells (from step 2.2.4).

2.3.2. Separately electroporate 300 ng of pK184-gene or pK184-gene mutant plasmids into 50 µL of electrocompetent XK1200 pOX38-Tc Δgene::Cm cells as per steps 2.1.4-2.1.7. All selective media should contain 10 µg/mL naladixic acid (Nal), 20 µg/mL Cm, and 50 µg/mL Km and be incubated at 37 °C.

2.3.3. Prepare an O/N of XK1200 pOX38-Tc Δgene::Cm+ pK184-gene recombinant cells by harvesting a single distinct colony of the cells with a sterile pipette or loop, and inoculating a 20 mL sterile LB media with Nal, 20 µg/mL Cm and 50 µg/mL Km. Keep the area sterile and work near a flame. Grow the cells O/N at 37 °C with shaking at 200 rpm.

2.3.4. Make 3-5 glycerol stocks from the O/N by mixing 500 µL of the O/N culture with 500 µL of sterile 100% glycerol (final 50% v/v) in sterile cryo-tubes. Store at -80 °C.

3. Conjugative Mating Assays

3.1. Conjugative Mating to generate XK1200 pOX38-Tc Δgene::Cm cells

3.1.1. Prepare a 20 mL sterile LB O/N culture of DY330R pOX38-Tc Δgene::Cm + pK184-gene cells by using a sterile pipette or loop to inoculate a 20 mL sterile LB containing 20 µg/mL Cm and 50 µg/mL Km with a glycerol stock or single colony on an agar plate. Grow at 32 °C with shaking at 200 rpm. Prepare the same for XK1200 cells in 20 mL sterile LB with 10 µg/mL Nal, growing at 37 °C.

3.1.2. Make 1:70 dilutions of each culture separately in 2 mL of sterile LB with the same antibiotic contents; add glucose to a final concentration of 100 mM to all donor cells. Grow cells to mid-log phase (OD_{600} 0.5-0.7) at 37 °C with shaking at 200 rpm.

3.1.3. Centrifuge (4000 x g for 5 min at 4 °C) to pellet cells, discard supernatant, wash once with cold sterile LB to remove antibiotics, and resuspend cells in 2 mL cold sterile LB.

3.1.4. Aliquot 100 µL of each culture into 800 µL of sterile LB media and allow them to mate at 32 °C for 1 h without shaking.

3.1.5. Vortex the cells for 30 s to disrupt the mating pairs and place them on ice for 10 min to prevent further mating.

3.1.6. Aliquot 100 µL of the cell mixture onto an agar plate containing 10 µg/mL Nal and 20 µg/mL Cm to select for XK1200 pOX38-Tc Δgene::Cm cells. Spread the cells using a sterile spreader. Keep the area sterile and work near a flame. Incubated the plate O/N at 37 °C upside-down.

3.1.7. Harvest a single colony of the new XK1200 pOX38-Tc *Δ*gene::Cm knockout strain using a sterile pipette or loop and grow in sterile LB with 20 µg/mL Cm, O/N at 37 °C with shaking at 200 rpm. Make 3-5 glycerol stocks from the O/N by mixing 500 µL of the O/N culture with 500 µL of sterile 100% glycerol (final 50% v/v) in sterile cryo-tubes. Store at -80 °C.

Note: The resultant cells are now able to be made competent for transformation with the pK184-gene constructs (protocols 1.3 and 1.4) for assessing the gene and its mutants on the ability to recover conjugative transfer in protocol 3.2.

3.2 Conjugative Mating Assay from XK1200 Donors to MC4100 Recipients

3.2.1. Prepare an O/N culture of XK1200 pOX38-Tc Δgene::Cm + pK184-gene cells in 20 mL of sterile LB with 20 µg/mL Cm, 50 µg/mL Km and MC4100 cells in 5 mL LB with 50 µg/mL streptomycin (Sm) using cells from a glycerol stock or single colony on an agar plate and sterile pipette or loop. Grow cultures at 37 °C with 200 rpm shaking.

3.2.2. Make 1:70 dilutions from each O/N culture separately in 2 mL of sterile LB with the same antibiotics. Add glucose to a final concentration of 100 mM to all donor cells. Grow cells to mid-log phase (OD_{600} 0.5-0.7) at 37 °C with shaking at 200 rpm.

3.2.3. Centrifuge (4000 x g for 5 min at 4 °C) to pellet cells, discard supernatant, wash once with cold sterile LB to remove antibiotics, and resuspend cells in 2 mL cold sterile LB.

3.2.4. In duplicate, aliquot 100 µL of each culture into 800 µL of sterile LB media and allow them to mate at 37 °C for 1 h without shaking.

3.2.5. Vortex the cells for 30 s to disrupt the mating pairs and place them on ice for 10 min to prevent further mating.

3.2.6. Using the mid-log cultures from step 3.2.2 and fresh sterile LB, prepare 6 serial dilutions of the donor and recipient cells from 10^{-2} to 10^{-7}.

3.2.7. On each of two halves of an agar plate containing 10 µg/mL Nal, 20 µg/mL Cm and 50 µg/mL Km, spot 10 µL aliquots of each dilution of XK1200 donor cells, as shown in **Figure 2**. Repeat for the dilutions of the recipient MC4100 cells on agar plates containing 50 µg/mL Sm. Incubate plates O/N at 37 °C.

3.2.8. Using the vortexed mixture from step 3.2.5 and fresh sterile LB, prepare 6 dilutions (10^{-2} to 10^{-7}) of the transconjugants. Select for the transconjugant MC4100 pOX38-Tc Δgene::Cm cells by spotting 10 µL aliquots of each of dilution on each half of agar plates containing 50 µg/mL Sm and 20 µg/mL Cm, as in **Figure 2.** Repeat for both duplicate mixtures. Keep the area sterile and work near a flame. Incubate plates O/N at 37 °C.

3.2.9. Determine the mating efficiency of each construct as described in protocol 3.3.

3.2.10. Repeat this protocol for all recovery plasmids to evaluate the effect of a particular mutation on the efficiency of conjugation.

3.3. Calculation of Mating Efficiency

3.3.1. Count the number of colonies from the same dilution spotting for each donor, recipient and transconjugant cells on their respective plates.

3.3.2. Count recipient colonies to test any bias that would result from having a larger number of transconjugants than recipients at that given dilution.

3.3.3. Calculate the mating efficiency as the number of transconjugant colonies divided by number of donor colonies. Multiply by 100 to obtain efficiency value per 100 donor cells.

REPRESENTATIVE RESULTS

The process of F plasmid-driven bacterial conjugation is a coordinated process that involves transfer proteins within the *tra* region of the F-plasmid that assembles a T4SS to facilitate pilus synthesis and conjugative DNA transfer. The protein TraF (GenBank accession # BAA97961; UniProt ID P14497) is required for conjugative F-pilus formation.[10,14,35–37] The protein contains a C-terminal thioredoxin-like domain, though it does not have the catalytic CXXC motif.[35,38] Although it has been predicted to interact with the TraH protein through its N-terminal domain,[39] a region shown to be more dynamic than its C-terminal domain,[37] not much else is known about the protein's structural features. To assess the functional aspects of the TraF protein in conjunction with structural studies, we first knocked out the *traF* gene on the pOX38-Tc via homologous recombination, generating the pOX38-Tc *ΔtraF*::Cm plasmid (**Table 1**) in *E. coli* DY330R cells.[33,34] Also generated was the pK184-TraF plasmid from *traF*-specific primers (**Table 2**) to provide recovery of conjugation and enable probing the protein's sequence. The transfer of the pOX38-Tc *ΔtraF*::Cm plasmid into XK1200 cells[40] from DY330R cells when pK184-TraF was provided *in trans* (transconjugants grown on 10 μg/mL Nal, 10 μg/mL Tc and 20 μg/mL Cm) indicates that (a) the *traF* knockout in pOx38-Tc *ΔtraF*::Cm provides an in-frame CAT cassette, and (b) that pK184-TraF can restore conjugative function.

A series of TraF mutants were generated for analysis using the conjugative mating assay[37] using XK1200 pOX38-Tc *ΔtraF*::Cm + pK184-TraF$_{\Delta X}$ cells and MC4100 cells[41] as donors and recipients, respectively. One representative mutant is TraF$_{55\text{-}247}$ (**Table 1**), an N-terminal deletion mutant that removes the region of the protein predicted to interact with TraH. When the full-length TraF protein is provided to the donor cells, conjugative transfer is restored (**Figure 2**), while providing the empty plasmid pK184 does not (**Table 3**). Similarly, conjugative function in the XK1200 donor cells is not restored when provided with plasmid pK184-TraF$_{55\text{-}247}$ (**Table 3**). This indicates that the truncated region of the protein is important for TraF's function within the conjugative apparatus, likely through interaction with TraH, and provides a region of the protein to target for further mutational analysis.

Figure 1. Schematic representation of the conjugative mating assay.
In Step 1, target genes are knocked out by homologous recombination in DY330R cells, an efficient recombination strain, using a PCR generated CAT cassette with overhangs homologous for the target gene. The resultant DY330R clone harbors the plasmid pOX38-Tc Δgene::Cm, which is unable to facilitate conjugation unless the knocked out gene is provided *in trans* via a recovery plasmid (pK184-gene). The resultant pOX38-Tc Δgene::Cm plasmid is transferred to a XK1200 strain (Step 2) for further assessment of the gene using mating assays with a MC4100 recipient (Step 3).

Figure 2. Mating assay to assess the target gene's function in conjugation.

Donor and recipient cells were *E. coli* XK1200 pOX38-Tc *ΔtraF*::Cm transformed with pK184-TraF and MC4100, respectively. The resulting transconjugants (MC4100 pOX38-Tc *ΔtraF*::Cm) grow on plates containing Sm and Cm, indicating successful recovery of conjugative function. The experiment is done in duplicate on a single agar plate, and serial dilutions are used in order calculate the mating efficiency of restorative gene mutants (**Table 2**).

Table 1: *E. coli* strains and plasmids used in this study.
†Cm, chloramphenicol; Km, kanamycin; Nal, nalidixic acid; Rif, rifampicin; Tc, tetracycline; Sm, streptomycin
‡All TraF deletion constructs (pK184-TraF$_{\Delta 1\text{-}\Delta 6}$) contain the 19 residue leader sequence to ensure proper localization to the periplasmic space.

Table 2: A list of primers used in this study.†
†Table adapted from Lento *et al*. 2016[18] with permission
*TraF overhanging regions are italicized. Restriction enzyme sites are underlined (HindIII: AAGCTT, EcoRI: GAATTC, NdeI: CATATG).

Table 3: Abolished mating efficiency by TraF deletion constructs.*
*Table adapted from Lento *et al*., 2016[18] with permission
†Donor cells were *E.coli* XK1200, with an average concentration of 3 x 10^7 cells/mL.
‡Recipient cells were *E. coli* MC4100. The number of transconjugants for the positive control is from a 10^{-5} dilution. 0 indicates no transconjugants from a 10^{-2} dilution.
§An average of two to four mating experiments were performed for each construct.
||Mating efficiency is defined as transconjugants per 100 donor cells. 0 mating efficiency indicates no transconjugants from a 10^{-2} dilution.

DISCUSSION:
Bacterial conjugation process provides a means by which bacteria can spread genes providing an evolutionary advantage for growth in challenging environments, such as the spread of antibiotic resistance markers. Because many of the conjugative plasmids are so large,[12] functional studies on the proteins involved in assembly of the transfer apparatus through mutation of target genes on the conjugative plasmid itself are unwieldy. The protocols detailed herein provide a means by which one can more readily assess the target gene of interest through the use of smaller, more manageable expression plasmids (**Figure 1**). We employ the F plasmid derivative pOX38-Tc (**Table 1**) to study F plasmid-mediated conjugation; other conjugative plasmids can be studied using the protocols detailed here and appropriate derivative plasmids. The mating assays outlined have been adapted from Frost and colleagues,[42] with some modifications. In previous studies,[8,33,42] the creation of the pOX38-Tc Δgene::Cm construct was achieved by cleaving the gene of interest with the appropriate restriction enzymes and inserting the amplified CAT cassette into pOX38-Tc.[42] In the current method, we employ homologous recombination in the recombineering *E. coli* strain DY330R[33,34] to knockout the target gene and replace it with the CAT cassette. This has an advantage of allowing the resultant DY330R strain harboring the pOX38-Tc Δgene::Cm construct to act as a control for the gene-specific knockout out F-T4SS mediated conjugative transfer via the recovery of transfer using the pK184-gene recovery plasmid. While it may be possible to generate knockouts using a CRISPER-Cas9 methodology,[43] we have not at this time explored this possiblity.

The process begins with the generation of a gene knockout in the F derivative plasmid pOX38-Tc (**Figure 1**). This is achieved via homologous recombination in DY330R cells (**Table 1**), other strains with similar features such as DY329, DY331 and DY378 can also be used. Primers are initially designed to PCR amplify the CAT cassette from the pBAD33 plasmid[32] and contain overhanging bases that are specific for the target gene (**Table 2**); the PCR product is then electroporated into DY330R cells harboring pOX38-Tc. Homologous recombination generates the knock-out plasmid pOX38-Tc Δgene::Cm where the CAT cassette is inserted in-frame within the target gene, effectively disrupting T4SS assembly and conjugation while providing Cm resistance. At the same time, the target gene is PCR amplified from pOX38-Tc and inserted into a small expression plasmid; in this protocol we use pK184 for constitutive expression, however one could chose a plasmid with inducible expression of the target gene if desired. The pK184-gene plasmid is then transformed into the DY330R pOX38-Tc Δgene::Cm cells, and these cells are then used as donors to transfer the pOX38-Tc Δgene::Cm construct via conjugation into XK1200 cells for mating assays. In the mating assays, the donor and recipient cells are XK1200 pOX38-Tc Δgene::Cm and MC4100, respectively. The pK184-gene recovery plasmid, as well as the series mutants of the target gene (deletions or point mutations), is provided to the donor cells to assess their ability to restore mating, and determine its efficiency, with MC4100 recipient cells (**Figure 2; Table 3**).

Critical to the procedure is the use of appropriate donor and recipient strains (**Table 1**), and the design of the primers used. For each primer designed, there are general guidelines that should be followed. While we strictly try to abide with 40-60% GC content, this may not always be possible. In such cases, it is the experimenter's discretion to test a primer with GC content slightly below or above this range. The melting temperature (T_m) of the forward and reverse primer must be similar, and the annealing temperature (T_a) value should always be lower than the T_m by 2-5 °C for PCR. The free energy available for allowing a hairpin to develop should be much lower than the T_a, while the homo- and hetero- dimerization T_m's must be very low (less than -10 kJ and 30 °C). Primers can be designed to probe deletions, insertions or point mutations as desired. It is of course critical that the resultant gene construct be amplified and ligated into the recovery plasmid in-frame such that it is properly expressed. Transformation of competent cells can be done via electroporation or heat shock using electrocompetent or chemically competent cells, respectively. We find that electroporation is more efficient for larger constructs such as pOX38-Tc and the homologous recombination oligonucleotide, while the smaller expression plasmids such as pK184-TraF can be readily transformed into cells using heat shock methods. Lastly, it is important to remember that there will be multiple antibiotics in use throughout the protocol, as both donor and recipient strains require different resistances that are different from the ones employed on the conjugative and recovery plasmids.

Aside from the mating assays techniques described here, there are other methods that are used to study bacterial conjugation, varying slightly in their approach. Horizontal gene transfer is a process where bacteria transfer a plasmid to a recipient cell, including interspecies recipients.[44] A study by Dahlberg and colleagues[44] for instance used bacterial conjugation to determine the extent of interspecies horizontal gene transfer. They utilized the incorporation of green fluorescent protein (GFP) into a plasmid cloned into KT2442 cells; the chromosomal *lac* I^q gene in the KT2442 cells repress GFP expression. When the plasmid carrying GFP is transferred to a

species without the *lac* I^q gene, fluorescence is observed.[44] Despite its limitation to provide protein specific function in various species, the interspecies conjugation experiment could possibly be coupled with the protocols presented here to make evolutionary predictions for protein-protein interactions between different species.

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The authors declare that they have no competing financial interests.

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Figure 1

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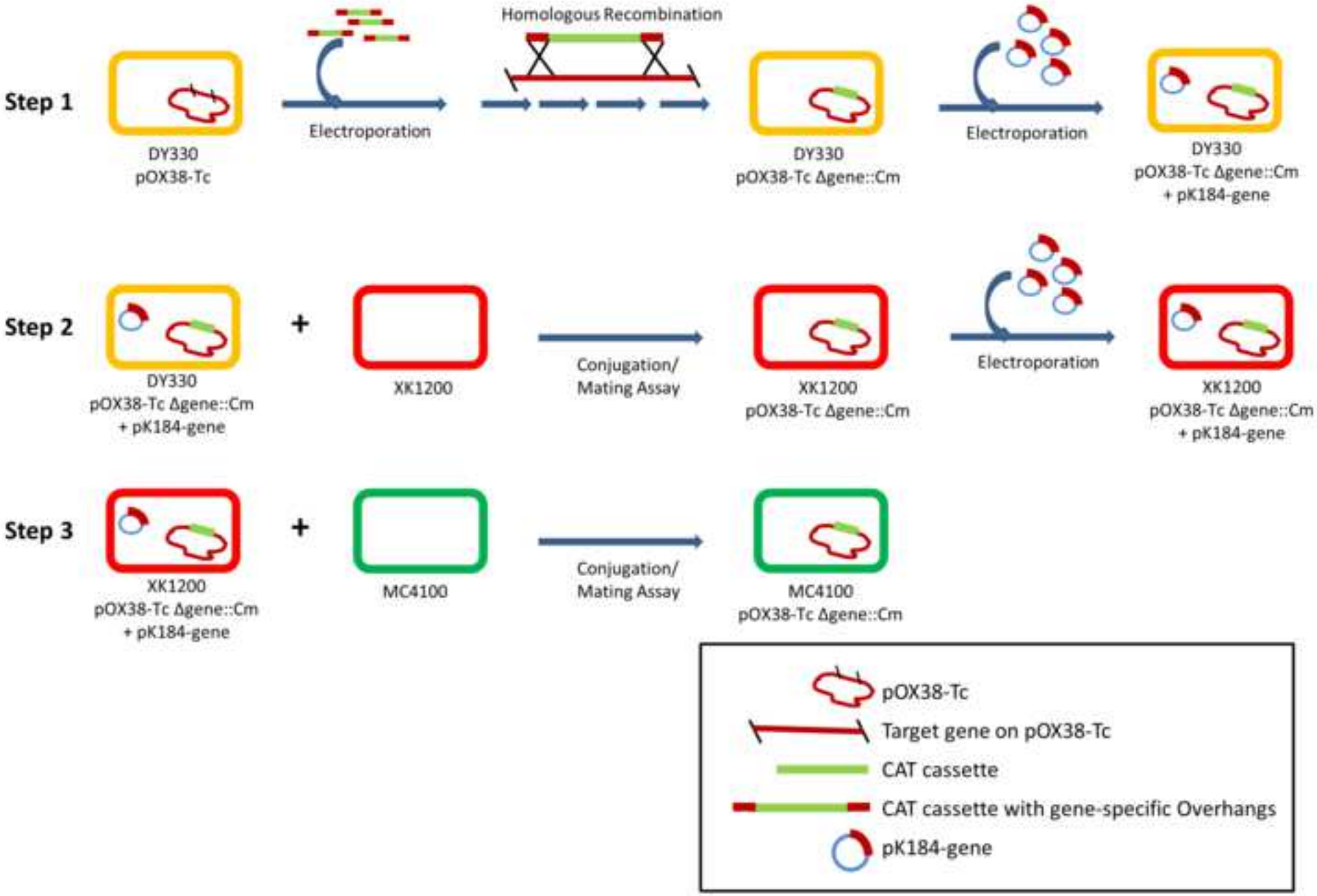

Figure 2

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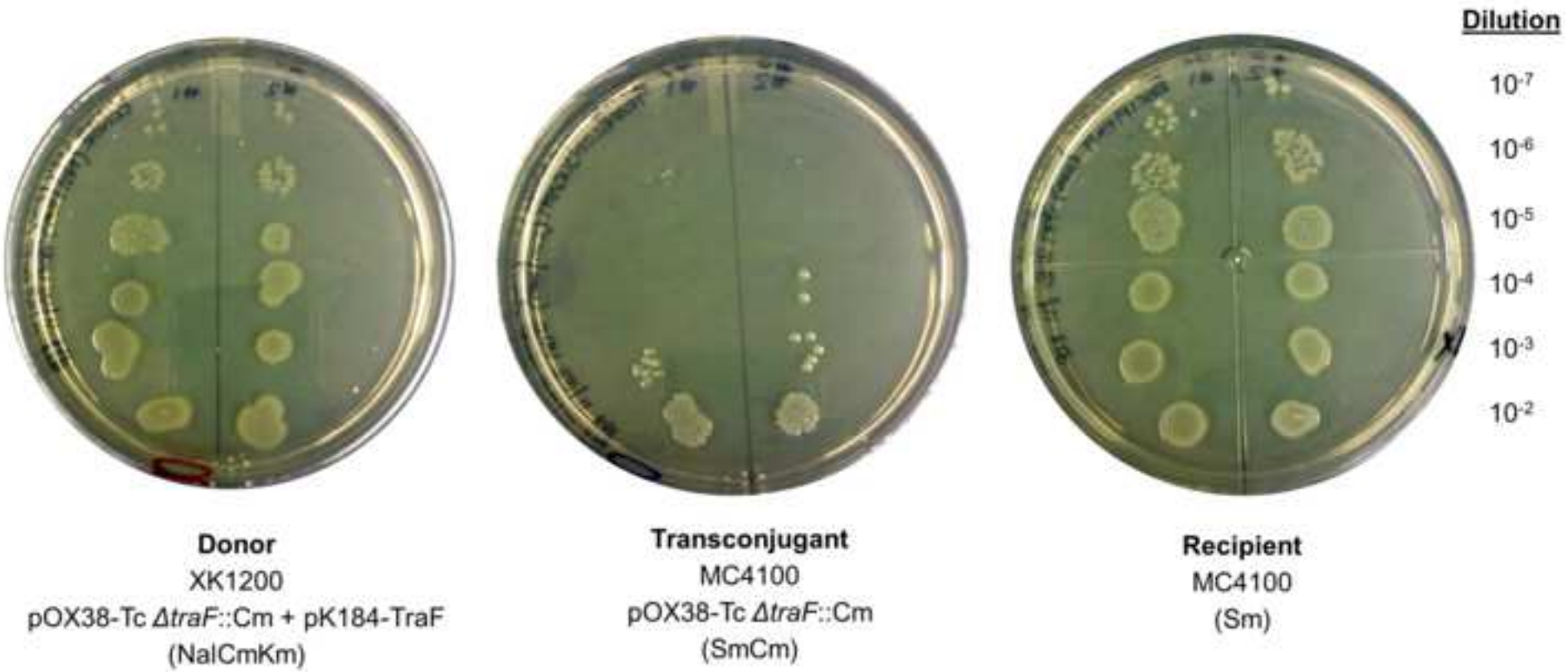

Table 1

Bacterial strain/Plasmid	Relevant Characteristics	Selective Marker(s)†	Reference
Bacterial Strains			
DY330R	W3110 *Δ* lacU169 *gal490* λc1857 *Δ* (cro-bioA) Rif^R	Rif	33,34
XK1200	F^- *lac Δ U124 Δ* (nadA *aroG gal att* λ *bio gyrA)*	Nal	40
MC4100	*araD139 Δ* (argF-lac)U169 *rpsL150 relA1 flbB3501 deoC1 ptsF25 rbsR*	Sm	41
Vectors and Constructs			
pBAD33	Plasmid for expression under from P_{araBAD}	Cm	32
pK184	2.4 kb cloning vector, p15a replicon	Km	31
pK184-TraF‡	F TraF from pOX38 in pK184	Km	35
pK184-$TraF_{56-247}$	F TraF aa 56-247 from pK184-TraF	Km	
Conjugative Plasmids			
pOX38-Tc	IncFI, Tra+, RepFIA+, f1 HindIII fragment of F, mini-Tn	Tc	29,30
pOX38-Tc *ΔtraF* ::Cm	pOX38-Tc with CAT inserted in *traF*	Tc Cm	35

†Cm, chloramphenicol; Km, kanamycin; Nal, nalidixic acid; Rif, rifampicin; Sm, streptomycin; Tc, tetracycline

‡All TraF constructs contain the 19 residue leader sequence to ensure localization to the periplasmic space.

Table 2

Construct	Primer*
TraF-Cm-For	5’- *GATCGAGGCTGGCAGTGGTATAACGAGAAAATAAATCCGAAGGA* - CTGTGACG GAAGATCACTTC -3’
TraF-Cm-Rev	5’- *TCTTCAGAAACGTTCAGGAACTGTTTTGCCAGGTCGTCCT* - CTTATTCAGGCGT AGCACCAG -3’
pK184-TraF-For	5’- TTTTTTGAATTCTATGAATAAAGCATTACTGCCAC -3’
pK184-TraF-Rev	5’- TTTTTTAAGCTTTAAAAATTGGGTTTAAAATCTTCAGAAA -3’
pK184-TraF$_{55-247}$-For	5’- TACGCATATGATGGCCGCACTGCAGACGG -3’
pK184-TraF$_{55-247}$-Rev	5’- TACGCATATGTCCTGACGCCGGAAAAATAAAGCAGCAGAGTAA -3’

†Table adapted from Lento et al. 2016[35] with permission

*TraF overhanging regions are italicized. Restriction enzyme sites are underlined (HindIII: AAGCTT, EcoRI: GAATTC, NdeI: CATATG)

Table 3

Donor Plasmid†	Recovery Plasmid	Transconjugants‡§ (cells ml^{-1})	Mating Efficiency§||
pOX38-Tc *ΔtraF* ::Cm	None	0	0
pOX38-Tc *ΔtraF* ::Cm	pK184	0	0
pOX38-Tc *ΔtraF* ::Cm	pK184-TraF	5×10^3	0.0167
pOX38-Tc *ΔtraF* ::Cm	pK184-$TraF_{55-247}$	0	0

*Table adapted from Lento *et al* . 2016[35] with permission

†Donor cells were *E.coli* XK1200, with an average concentration of 3×10^7 cells ml^{-1}

‡Recipient cells were *E. coli* MC4100. The number of transconjugants for the positive control is from a 10-5 dilution. 0 indicates no transconjugants from a 10-2 dilution.

§An average of two to four mating experiments were performed for each construct.

||Mating efficiency is defined as transconjugants per 100 donor cells. 0 mating efficiency indicates no transconjugants from a 10-2 dilution

Click here to download Excel Spreadsheet- Table of Materials/Equipment Materials.xlsx

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
GeneJet Plasmid Mini-Prep Kit	Fisher Scientific	K0503	
GeneJet Gel Extraction Kit	Fisher Scientific	K0692	
GeneJet PCR Purification Kit	Fisher Scientific	K0702	
Q5 Site-Directed Mutagenesis Kit	New England Biolabs	E0554S	
Broad Range DNA Ladder	New England Biolabs	N0303A	
Petri Dishes	Fisher Scientific	FB0875713	
Electroporator	Eppendorf	4309000027	
Electroporation cuvettes	Fisher Scientific	FB101	Cuvettes have a 1 mm gap.
Enzymes			
AvaI	New England Biolabs	R0152S	
EcoRI	New England Biolabs	R0101S	
HindIII	New England Biolabs	R0104L	
NdeI	New England Biolabs	R0111S	
Phusion DNA Polymerase	New England Biolabs	M0530L	
T4 DNA Ligase	New England Biolabs	M0202S	
DpnI	New England Biolabs	R0176S	
Antibiotics			**Final Concentrations**
Chloramphenicol (Cm)	Fisher Scientific	BP904-100	20 µg/mL
Kanamycin (Km)	BioBasic Inc.	DB0286	50 µg/mL
Nalidixic acid (Nal)	Sigma-Aldrich	N8878-25G	10 µg/mL

Rifampicin (Rif)	Calbiochem	557303	20 µg/mL
Tetracycline (Tc)	Fisher Scientific	BP912-100	10 µg/mL
Streptomycin (Sm)	Fisher Scientific	BP910-50	50 µg/mL

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Title of Article: Conjugative Mating Assays for Sequence-Specific Analysis of Transfer Proteins Involved in Bacterial Conjugation

Author(s): Fettah Erdogan, Cristina Lento, Ayat Yaseen, Roksana Nowroozi-Dayeni, Sasha Kheyson and Gerald F. Audette

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August 9, 2016

Re: Revisions to JoVE Manuscript 54854_R1_050416

To Whom It May Concern:

Thank you for your consideration of our manuscript, "Conjugative Mating Assays for Sequence-Specific Analysis of Transfer Proteins Involved in Bacterial Conjugation", for publication in the *Journal of Visualized Experiments* (JoVE). We would also like to thank the editor and reviewers for their excellent comments and insights. We have extensively revised our manuscript as per the reviewer comments, which are outlined below. In the process of revising our manuscript, we have also carefully reviewed and revised any grammatical errors. Several points in particular were noted by the reviewers:

Editor

1. The revised document has been revised using track changes as requested.
2. Grammar and Spelling – we have taken the opportunity to carefully review the spelling and grammar during our revisions as noted by the editor. Thank you.
3. We have removed the italics for "gene" as requested, confirmed that all degrees are denoted by the "degree" symbol and included DOI for references where possible.
4. We have changed 3.1.8 to a note as it is more appropriate that way and changed instances of "your/you"
5. We have removed highlights from the sections/lines noted and included in highlight the counting and calculation steps.
6. Additional details requested – plasmids have been cited, both in the text and Table 2; we have include example thermocycler settings as a note under protocol 1.2.7.3; we have included antibiotic concentrations where appropriate in the text of the protocol.
7. We have included our copyright permissions as requested.
8. Please note that we have numbered out tables as follows: Table 1 – Materials, Table 2 – *E. coli* strains and plasmids used in this study, Table 3 - A list of primers used in this study, and Table 4 - Abolished mating efficiency by TraF deletion construct. Please advise if this is not as the tables would be viewed in the final publication as we can renumber accordingly.

Reviewer 1

- We thank the reviewer for their excellent comments and insights. We have rewritten the introduction to be more general in terms of being more "big picture" as requested while introducing the F plasmid as an example of a larger conjugative plasmid (thank you indeed for this comment, it makes the framing of the protocol more approachable). As we note in our response to Reviewer 2 below, this manuscript has been written to make these methods more approachable for researchers who are coming to conjugative systems from a more structural standpoint and may not have the extensive background of plasmid biology as other groups.
- Regarding Reviewer 1's specific comments:
  1. We have amended the statement on line 119 as requested.

2. The strain used is DY330R – We apologize for the confusion and included strain DY300 for completeness in the original submission. We have clarified this in the text and Table 2 as requested.
3. The recombineering protocols as noted for DY330R – We apologize for the misprint of 37 °C temperature versus 32 °C as noted by the reviewer. We have also included a note after step 2.1.7 as requested regarding growth conditions of DY330R.
4. Selective markers - We thank the reviewer for highlighting this issue. We agree with the reviewer that inclusion of extra selective pressure is unduly challenging to the cells, have rechecked all our experiments to confirm that the extra selective pressures are not required and revised our complete protocol accordingly.
5. Figure 1 – We have revised the figure as requested to be less confusing to the reader.

Reviewer 2

- We thank the reviewer for their time and efforts in review of our manuscript. In response to their concern regarding existing protocols, we do agree that many of the protocols are available. However, the purpose of our manuscript was to provide a description of the conjugative mating assay for coupling to structural studies such as X-ray crystallography, which is the major focus of our lab. The conjugative mating assay may be more routine for microbiology groups, in particular those that are well-versed in plasmid biology. However we have found that a detailed protocol, such as a JoVE article, would be of significant benefit for labs such as ours, whose main focus is structural studies of conjugative systems and wish to couple these analyses with functional studies of the component proteins. Accordingly we believe that our manuscript provides benefit for the scientific community.

Reviewer 3

- We thank the reviewer for their efforts and excellent comments, and have addressed them as noted below:
  1. We have revised the text noted for lines 59, 64, 71 and 627 as requested.
  2. Regarding the text referring to "connecting the cytoplasms of donor and recipient cells". We agree that it is indeed still unclear if the mating pair formation complex assembled by the conjugative T4SS does indeed physically connect the respective cytoplasms via a lumen of the pilus. Although Silverman's group noted during the cryo-EM analysis of the F-pilus (Wang et al. J. Mol. Biol. (2009) 35, 22-29) that the transfer of DNA was likely through the lumen of the pilus based on the calculated size of the pilus lumen, which is where our original statement originated, a clear connection of the donor and recipient cytoplasms has yet to be established. We have therefore revised our language accordingly to remove this statement/inference.
  3. Lines 83-84 – we have revised our text as noted by the reviewer regarding high mutation rates and conjugative mediated evolution.
  4. We have revised our introduction and discussion to clearly indicate that the methods described in the protocol are for larger conjugative plasmids. We agree with the reviewer that the Smillie et al. (2010) paper provided an excellent discussion of mobile plasmids and that they identified a large range of sizes in mobile plasmids. We include this in our introduction and discussion, and more clearly articulate that the protocol we describe, in particular the homologous recombination portion, are for these larger conjugative plasmids that are less amenable to standard recombination methods as applied for smaller plasmids.

Thank you again for your consideration of our manuscript for publication in *JoVE*. If there are any questions or concerns with the manuscript, please do not hesitate to contact me by email at audette@yorku.ca.

Sincerely,

Gerald F. Audette