

Conjugation Assay for Testing CRISPR-Cas Anti-plasmid Immunity in Staphylococci

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[Abstract] CRISPR-Cas is a prokaryotic adaptive immune system that prevents uptake of mobile genetic elements such as bacteriophages and plasmids. Plasmid transfer between bacteria is of particular clinical concern due to increasing amounts of antibiotic resistant pathogens found in humans as a result of transfer of resistance plasmids within and between species. Testing the ability of CRISPR-Cas systems to block plasmid transfer in various conditions or with CRISPR-Cas mutants provides key insights into the functionality and mechanisms of CRISPR-Cas as well as how antibiotic resistance spreads within bacterial communities. Here, we describe a method for quantifying the impact of CRISPR-Cas on the efficiency of plasmid transfer by conjugation. While this method is presented in *Staphylococcus* species, it could be more broadly used for any conjugative prokaryote.

Keywords: Conjugation, CRISPR-Cas, Plasmids, *Staphylococcus*, Antibiotic resistance, Horizontal gene transfer

[Background] CRISPR-Cas (Clustered, regularly interspaced, short palindromic repeats-CRISPR associated) is a prokaryotic adaptive immune system found in almost 90% of sequenced archaea and about 40% of bacteria (Makarova *et al.*, 2015). These systems recognize and destroy nucleic acid invaders in a sequence-specific manner (van der Oost *et al.*, 2014). A CRISPR locus typically contains an array of short DNA repeats (~35 nucleotides in length) separated by equally-short unique sequences called spacers, which are often derived from mobile genetic elements. The repeats and spacers are transcribed and processed into small CRISPR RNAs (crRNAs) that each specifies a single target. crRNAs assemble with one or more Cas proteins to form an effector complex that recognizes and degrades nucleic acids that bear a sequence, called a protospacer, complementary to the crRNA. Depending on the CRISPR-Cas type present in an organism, other limitations exist on target recognition, such as the presence of a short protospacer-adjacent motif (PAM) required for targeting by Type I and II systems (Mojica *et al.*, 2009) or the requirement in Type III systems for specific base-pair mismatches between the crRNA and the target (Marraffini and Sontheimer, 2010). Nucleic acid invaders targeted by CRISPR-Cas are typically bacteriophages or mobile genetic elements such as plasmids or transposons that often carry virulence factors, including antibiotic resistance genes and toxins (Novick, 2003; Liu *et al.*, 2016). CRISPR-Cas is thus a potential major barrier to the spread of virulence factors within prokaryotes and has been shown to prevent conjugative transfer of antibiotic resistance plasmids within human clinical isolates of *Staphylococcus* species (Marraffini and Sontheimer, 2008). Some staphylococci, including human pathogenic isolates of *S. epidermidis* and *S. aureus*, the two *Staphylococcus* species most commonly found in human infections, carry

CRISPR-Cas systems (Cao *et al.*, 2016; Li *et al.*, 2016). Within these strains, multiple CRISPR spacers have been found that naturally bear homology to mobile staphylococcal plasmids, indicating that these organisms are capable of using CRISPR-Cas to limit the spread of virulence factors (Samai *et al.*, 2015; Li *et al.*, 2016). Investigations of the effect of mutations within the *cas* genes and the repeat-spacer array in *Staphylococcus* on anti-plasmid immunity have provided key insights into the function and mechanisms of Type III-A CRISPR-Cas in *S. epidermidis* RP62a (Hatoum-Aslan *et al.*, 2011 and 2014; Maniv *et al.*, 2016). Notably, similar assays have been used for mechanistic CRISPR-Cas studies in other organisms, including *Enterococcus faecalis*, *Escherichia coli*, and *Listeria monocytogenes* (Richter *et al.*, 2014; Sesto *et al.*, 2014; Price *et al.*, 2016). Other methods of quantifying CRISPR anti-plasmid immunity, namely transformation via electroporation, have been used (Cao *et al.*, 2016); however, this method cannot be used in non-competent or weakly/selectively competent organisms such as *L. monocytogenes* and many strains of *Staphylococcus* (Monk *et al.*, 2012; Sesto *et al.*, 2014). In these organisms, conjugation assays provide not only a viable alternative, but also a more physiologically relevant means of testing CRISPR-Cas anti-plasmid immunity. Described below is a quantitative method to determine the efficacy of CRISPR-mediated interference of the transfer of conjugative plasmid pG0400 between *S. aureus* and *S. epidermidis*. While this protocol focuses on staphylococci, it could be adapted for any prokaryotes capable of conjugation.

Materials and Reagents

1. 15 ml centrifuge tubes (VWR, catalog number: 21008-216)
2. 1.7 ml microcentrifuge tubes (VWR, catalog number: 87003-294)
3. 0.45 µm membrane filters, 25 mm (EMD Millipore, catalog number: HAWP02500)
4. 50 ml centrifuge tubes (VWR, catalog number: 21008-242)
5. Pipette tips with filter, 100-1,000 µl (VWR, catalog number: 89003-060)
6. Pipette tips with filter, 1-200 µl (VWR, catalog number: 89003-056)
7. Pipette tips with filter, 1-40 µl (VWR, catalog number: 89003-048)
8. 100 x 15 mm Petri dishes (VWR, catalog number: 25384-088)
9. 0.2 ml PCR strip tubes (VWR, catalog number: 20170-004)
10. 1 mm path length cuvettes (VWR, catalog number: 97000-586)
11. Recipient *S. epidermidis* RP62a (Christensen *et al.*, 1985), bearing a CRISPR-Cas system (see Note 1) (ATCC, catalog number: 35984)
12. Donor *S. aureus* RN4220 (Kreiswirth *et al.*, 1983), bearing the conjugative plasmid pG0400 (Morton *et al.*, 1995) (see Note 2) (ATCC, BEI Resources, catalog number: NR-45913)
13. Negative control *S. epidermidis* LAM104 (Marraffini and Sontheimer, 2008), lacking the CRISPR repeat-spacer array (see Note 3)
14. Mupirocin (The United States Pharmacopeial Convention, catalog number: 1448901)
15. Brain-heart infusion broth (BD, BBL, catalog number: 211060)
16. Brain-heart infusion agar (BD, Difco, catalog number: 241830)

17. Tryptic soy broth (BD, Bacto™, catalog number: 211822)
18. Neomycin sulfate (AMRESCO, catalog number: 0558-25G)
19. Brain-heart infusion broth (see Recipes)
20. Brain-heart infusion agar (see Recipes)
21. Tryptic soy broth (see Recipes)

Equipment

1. Micropipettes set (Eppendorf, model: Research® Plus)
2. 37 °C incubator (Thermo Fisher Scientific, Thermo Scientific™, model: Heratherm™ Advanced Protocol Microbiological Incubators)
3. 37 °C incubated shaker capable of 160 rpm (Eppendorf, model: I26)
4. Autoclave (Getinge)
5. Spectrophotometer (GE Healthcare, model: UltroSpec 10)
6. Microcentrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: Heraeus™ Pico™ 17)
7. Digital vortex mixer (VWR, model: Advanced Heavy-Duty Vortex Mixer)
8. Inoculating wire loop
9. Forceps
10. Magnetic stir plate

Procedure

1. Day 1. Preparing strains
 - a. Streak a brain-heart infusion (BHI) agar plate containing 15 µg/ml neomycin with *S. epidermidis* RP62a (from here called 'recipient') from a -80 °C freezer stock to obtain single colonies. Incubate overnight at 37 °C.
Note: In the same manner, streak out the negative control strain, S. epidermidis LAM104.
 - b. Inoculate *S. aureus* carrying pG0400 (here onward called 'donor') from a freezer stock into 2 ml of tryptic soy broth (TSB) containing 5 µg/ml mupirocin. Incubate with shaking at 37 °C overnight.
Note: While both S. epidermidis and S. aureus strains may be propagated in either BHI or TSB, the media indicated for each strain in this protocol are consistent with the growth media used in the original protocol demonstrating anti-plasmid immunity by the Type III-A CRISPR-Cas system in S. epidermidis RP62a (Marraffini and Sontheimer, 2008).
2. Day 2. Growing colonies and filter mating
 - a. After at least 16 h of incubation, large (> 1 mm in diameter) individual recipient colonies should be seen. Inoculate the entirety of 3 separate colonies into 15-ml centrifuge tubes containing 2 ml of BHI with 15 µg/ml neomycin.
Notes:

- i. Also, inoculate any controls included in the experiment in triplicate.
- ii. At the same time, add 50 μ l of the overnight donor culture to 5 ml of TSB with 5 μ g/ml mupirocin in a 15-ml centrifuge tube.
- b. Grow both the donor and recipient at 37 °C with shaking.
Note: At the same time, place one BHI plate per 3 growing recipient cultures into the 30 °C incubator to pre-warm (see Note 4).
- c. After ~6 h, check the OD₆₀₀ of both the recipient and donor strains.
 - i. Add 900 μ l of BHI broth into a spectrophotometer cuvette. Add 100 μ l of growing culture of one of the recipient cultures. Mix well.
 - ii. After blanking with BHI, measure and record the OD₆₀₀ of this 1:10 dilution of the growing culture.
 - iii. Repeat steps 2c i and 2c ii with the remaining recipient cultures.
 - iv. Repeat steps 2c i and 2c ii for the donor culture, substituting TSB for BHI.
 - v. OD₆₀₀ of all culture dilutions needs to be greater than 0.20 before proceeding. If the OD₆₀₀ are not yet high enough, return to the shaker and let grow a while before checking the OD₆₀₀ again.
- d. Calculate the amount of donor and recipient to combine to achieve a 1:4 ratio (recipients:donors) for filter mating, as in the example in Table 1.
Note: Divide 200 by the measured OD₆₀₀ found in step 2c for each culture. At an OD₆₀₀ of 1, there are approximately 8 x 10⁸ cfu/ml of the donor and 2 x 10⁸ cfu/ml of the recipient. This calculation allows for standard numbers of donors (approximately 1.6 x 10⁹ cfu) and recipients (approximately 4 x 10⁸ cfu) to be combined in all samples that are tested.

Table 1. Sample calculations to determine volumes of cultures to combine for filter mating

Culture	OD ₆₀₀ (1:10 dilution)	200/OD ₆₀₀ (Volume to add, μ l)
RP62a (1)	0.42	476
RP62a (2)	0.42	476
RP62a (3)	0.41	488
LAM104 (1)	0.44	455
LAM104 (2)	0.39	513
LAM104 (3)	0.44	455
RN4220/pG0400	0.56	357

- e. Into a 1.7 ml microcentrifuge tube, combine the above-calculated volume of the first, undiluted, recipient culture with the calculated volume of the undiluted donor.
Note: Repeat until all donor-recipient pairs are combined into separate microcentrifuge tubes.
- f. Wash the combined cultures to remove the antibiotics by centrifuging the microcentrifuge tubes at 6,200 x g for 2 min in a table-top centrifuge. Pipette off the supernatant and resuspend the pellets in 1 ml of BHI without antibiotics. Repeat the centrifugation.

- g. Using sterile forceps, place one 0.45 μm membrane filter for each donor-recipient pair onto a BHI plate containing no antibiotics, with up to 3 filters on a single plate (Figure 1).

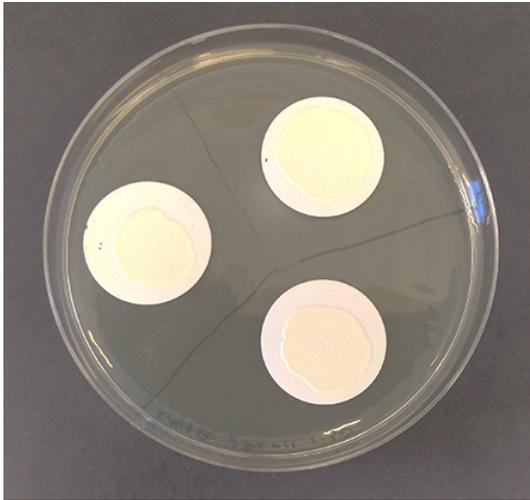


Figure 1. Sample plate for filter mating showing a plate with three membrane filters covered in overnight growth in the center of each filter

- h. Remove the suspension from each microcentrifuge tube containing the donor and recipient combination. Resuspend the pellet in 100 μl of BHI broth and carefully pipet it onto the center of the membrane filter, being careful to not let any of the suspension fall off the membrane, as in Figure 1. It is crucial to keep all of the bacterial suspension on the filter, as this assay is quantitative and requires total recovery of all the cells.
- i. Repeat this with each microcentrifuge tube, placing the resuspension of each culture onto its own filter.
- j. Cover the plates and leave on the benchtop until the cell suspension has fully dried.
- k. Incubate the plates upside-down in a 30 $^{\circ}\text{C}$ incubator overnight.
3. Day 3. Serially diluting and plating recipients and transconjugants
- a. Warm one BHI agar plate containing neomycin (15 $\mu\text{g}/\text{ml}$) and one BHI agar plate containing both neomycin (15 $\mu\text{g}/\text{ml}$) and mupirocin (5 $\mu\text{g}/\text{ml}$) for each filter from the previous night.
- b. In 50-ml centrifuge tubes (one for each filter), add 3 ml of BHI broth without antibiotics.
- c. Using sterile forceps, pick each filter off the plate and place it into a 50-ml tube prepared in step 3b.
- d. Vortex each tube until the cells on the filter have been fully resuspended and no growth is visible on the filter.
- e. Serially dilute the cell suspension
- i. Into each of the 8 tubes in a strip of PCR tubes, add 90 μl BHI broth.
 - ii. Remove 10 μl of the bacterial suspension from step 3d and add it to the first PCR strip tube, pipetting up and down repeatedly to resuspend (this is the 10^{-1} dilution).

- iii. Using a new pipette tip, remove 10 μ l from the 10⁻¹ dilution and add it to the second PCR tube, pipetting up and down repeatedly to resuspend (this is the 10⁻² dilution).
- iv. Repeat step 3e iii to create dilutions all the way out to 10⁻⁸.
Note: Alternative methods of performing a series of 10-fold serial dilutions, such as using a multichannel pipette in a 96-well plate may be used instead.
- f. Spot 10 μ l of each dilution onto a pre-warmed BHI plate containing neomycin and another containing neomycin and mupirocin (see Note 5). All dilutions to be plated for recipient colony counting should be plated on the same plate, as in Figure 2A, and all dilutions to be plated for transconjugant colony counting should be plated on an additional plate, as in Figure 2B.
Note: Tilt the plate at a 45° angle until the drop of the dilution almost reaches the edge of the plate, then set it flat on the benchtop and let dry.

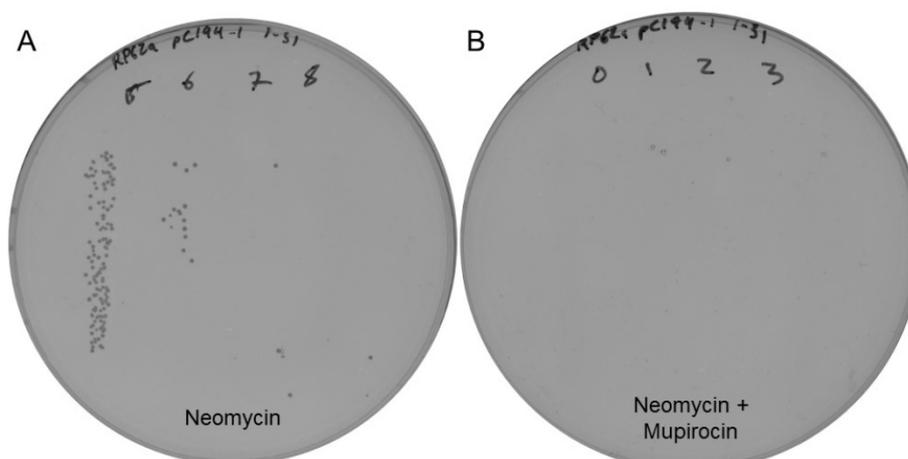


Figure 2. Sample plates from a conjugation assay using a strain of RP62a, showing recipients (A. 1.5 x 10⁹ cfu/ml) and transconjugants (B. 0 cfu/ml)

- g. Repeat steps 3e and 3f for each filter.
- h. Let the plates dry while covered on the benchtop, then incubate at 37 °C overnight.
4. Day 4. Recipient and transconjugant counting
 - a. Count the number of recipients (colonies on plates containing only neomycin) for each filter from Day 2, as shown in Figure 2A.
 - i. Determine the number of colonies in the highest dilution where the colonies are still countable.
 - ii. Multiply the number of colonies by 100 and by the dilution factor (10¹ for the 10⁻¹ dilution, 10² for the 10⁻² dilution, etc.) to obtain the number of colony forming units per milliliter.
 - b. Count the number of transconjugants (colonies on plates containing neomycin and mupirocin) for each filter from Day 2, as in step 4a and shown in Figure 2B.

- c. Calculate the conjugation efficiency (see Data analysis).

Data analysis

1. Calculation of conjugation efficiency

- a. Conjugation efficiency is a measure of the number of transconjugants produced per each initial recipient cell. It can be simply calculated as follows, with an example shown in Table 2:

$$Efficiency = \frac{\text{Number of Transconjugants } (\frac{cfu}{mL})}{\text{Number of Recipients } (\frac{cfu}{mL})}$$

Table 2. Calculation of conjugation efficiency of a sample conjugation assay

Strain	Transconjugants (cfu/ml)	Recipients (cfu/ml)	Conjugation Efficiency
RP62a (1)	1 x 10 ³	7 x 10 ⁹	1 x 10 ⁻⁷
RP62a (2)	2 x 10 ²	1 x 10 ⁹	2 x 10 ⁻⁷
RP62a (3)	0	4 x 10 ⁹	0
LAM104 (1)	1.3 x 10 ⁵	3 x 10 ⁹	4 x 10 ⁻⁵
LAM104 (2)	9 x 10 ⁴	3 x 10 ⁹	3 x 10 ⁻⁵
LAM104 (3)	9 x 10 ⁴	4 x 10 ⁹	2 x 10 ⁻⁵

- b. A high efficiency indicates an inactive CRISPR system, while a lowered efficiency indicates active CRISPR defense.
- i. A 100 to 1,000-fold decrease in conjugation efficiency is typically seen in the presence of a CRISPR system as compared to a strain without a CRISPR system.

2. Data presentation

Data is typically shown in a bar graph showing the number of both recipients and transconjugants in cfu/ml (colony-forming units per milliliter) on a logarithmic scale, with the calculated conjugation efficiencies accompanying the graph, as in Figure 3.

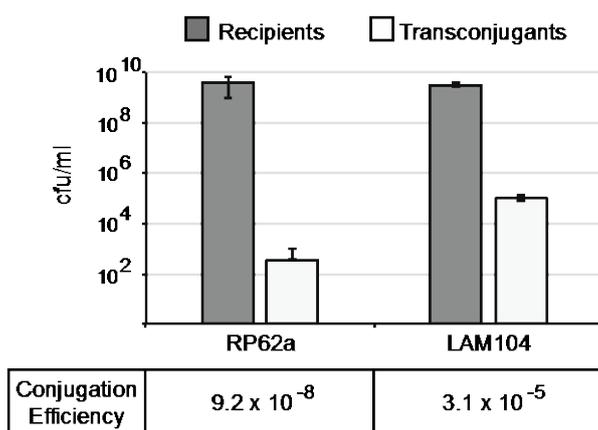


Figure 3. Sample graph of data from a conjugation assay using *S. epidermidis* RP62a and LAM104 as recipients. Error bars indicate the standard deviation of triplicate measurements.

Notes

- Other strains can be used as the recipient strain in this assay. *S. epidermidis* RP62a naturally carries a CRISPR spacer that targets the *nickase* gene found on conjugative staphylococcal plasmids such as pG0400 (Marraffini and Sontheimer, 2008). Other CRISPR-containing prokaryotes can be used if they are amenable to receiving conjugative plasmids. An additional important consideration in recipient selection is to make sure that the recipient has a selectable marker that differentiates it from the donor strain. In the case of conjugation assays with *S. epidermidis*, *S. epidermidis* RP62a is resistant to neomycin, so this resistance can be used to select only for transconjugants and not the donor strain itself on Day 3. Recipient strains that do not have differing antibiotic susceptibilities naturally can be transformed with a plasmid harbouring an additional resistance marker (such as pC194 [Ehrlich, 1977] for *Staphylococcus*). Whichever plasmid is chosen, ensure its origin is compatible with the conjugative plasmid to allow for maintenance and selection of both plasmids in the transconjugants. If using different selective markers, change antibiotics as appropriate for culturing and plating.
- Other strains of *S. aureus* can be used as the donor strain in this assay, as can additional conjugative plasmids. The donor strain needs to be able to perform conjugation with the recipient strain, and RN4220 is very convenient for this conjugation assay as it can perform conjugation with *S. epidermidis* RP62a and lacks a CRISPR system, allowing for ready uptake and maintenance of plasmids such as pG0400 that could be degraded in a CRISPR-bearing strain. Additional conjugative plasmids can also be used, depending on the CRISPR spacers found within the recipient strain. If testing to see if CRISPR functions in anti-plasmid activity in the recipient strain, a conjugative plasmid that bears a protospacer complementary to a spacer found within the CRISPR array of the recipient must be used. In the case of RP62a, pG0400 naturally has this protospacer, but in many systems, it will likely be necessary to engineer a

conjugative plasmid with a protospacer. If using a different conjugative plasmid, change antibiotics as appropriate for culturing and plating. Additionally, if engineering a conjugative plasmid, be sure to follow the rules required for CRISPR targeting in the specific system to be tested, such as following the PAM requirement for Types I and II systems (Mojica *et al.*, 2009) and the required mismatches that permit interference by Type III-A systems (Marraffini and Sontheimer, 2010).

3. LAM104 is an engineered derivative of RP62a that lacks the repeat-spacer array, preventing CRISPR-Cas from functioning (Marraffini and Sontheimer, 2008). If using a strain other than RP62a, it is recommended to use a negative control in which CRISPR-Cas does not function. Suitable negative controls include 1) the use of a recipient strain that lacks a crucial component of CRISPR-Cas or 2) the use of a donor strain that carries a conjugative plasmid lacking an appropriate protospacer. *S. epidermidis* LAM104 is not available commercially, but can be made available from the Hatoum-Aslan laboratory on request.
4. With *S. epidermidis* RP62a, increased background counts of recipients have been observed on Day 4 if filter mating occurs at 37 °C. To obtain a cleaner result, a 30 °C incubation is routinely used, although this can be modified to suit the growth requirements of any organism.
5. With *S. epidermidis* strains as recipients, transconjugants are typically seen in a concentration from 10⁰-10⁵ cfu/ml, and therefore only the 10⁰ to 10⁻³ dilutions are typically plated on the BHI plates containing neomycin and mupirocin to obtain colony counts. Recipients are usually around 10⁹ cfu/ml and thus usually only the 10⁻⁵ to 10⁻⁸ dilutions are plated on the BHI plates containing neomycin to obtain colony counts.

Recipes

1. Brain-heart infusion broth
 - a. Dissolve 19.5 g brain-heart infusion in 500 ml deionized (DI) water
 - b. Autoclave at 121 °C for 30 min and store at room temperature
2. Brain-Heart infusion agar
 - a. Dissolve 29 g brain-heart infusion agar in 500 ml DI water
 - b. Autoclave at 121 °C for 30 min and stir on a magnetic stir plate until it has cooled enough to be comfortable to the touch, then add antibiotics and pour plates immediately (20 plates per 500 ml)
Note: Media can also be stored at room temperature and later heated in the microwave to liquefy and pour.
3. Tryptic soy broth
 - a. Dissolve 15 g of tryptic soy broth in 500 ml DI water
 - b. Autoclave at 121 °C for 30 min and store at room temperature

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