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A novel high-throughput screening method for identifying compounds that inhibit plasmid conjugation

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ABSTRACT

Plasmid conjugation is an important contributing factor to the spread of antibiotic resistance among bacteria, posing a significant global health threat. Our method introduces an innovative high-throughput screening approach to identify compounds that inhibit or reduce conjugation, addressing the need for new strategies against the spread of antimicrobial resistance.

Using *Escherichia coli* strains as donor and recipient, we screened 3500 compounds from a library provided by ABAC Therapeutics. Each 96 –well plate was loaded with 88 different compounds and bacterial cultures. Every plate also included negative and positive controls of conjugation. After an hour, cultures from wells were spotted on agar plates and assessed visually. Compounds that showed a visible effect on conjugation were retested. Six compounds targeting conjugation were found, showing promise for further analysis.

Specifications table

Subject area:	Immunology and Microbiology
More specific subject area:	Bacteriology
Name of your protocol:	Compound Library Screening
Reagents/tools:	Luria-Bertani (LB) broth (Invitrogen, Barcelona, Spain), Cefotaxime and rifampicin (Sigma, Copenhagen, Denmark), 96-well plate
Experimental design:	A compound library is loaded into 96-well plates, followed by the recipient and donor strains. Plates included different controls. After one hour of incubation, cultures from wells were spotted on agar plates. Spots were assessed visually compared to controls in order to identify compounds with potential effect on plasmid conjugation.
Trial registration:	Not Applicable
Ethics:	Not Applicable
Value of the Protocol:	This method: • Provides a rapid and easy way for identifying potential inhibitors of plasmid conjugation from a large compound library • Is a cost effective high-throughput screen, with limited equipment requirements.

Description of protocol

Background

Plasmid conjugation is one of the most important mechanisms for the transfer of genetic material from one bacterial cell to another using plasmids [1]. However, given that antibiotic resistance genes are frequently found on plasmids, conjugation has been

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a significant contributor to the spread of antibiotic resistance [2–6]. We developed a novel method to screen for compounds that could reduce or inhibit plasmid conjugation. This method provides a fast, simple way to detect these compounds from a large library, which would be the first step to find new drugs.

Method details

Bacterial strains and culture conditions

The donor strain used in this experiment is *E. coli* MG1655/pTF2 [7]. pTF2 is a 93-kb conjugative plasmid belonging to the IncI1 plasmid family carrying the gene $bla_{CTX\text{-}M\text{-}1}$ conferring resistance to cefotaxime. The recipient strain is *E. coli* J53-2 [8], resistant to rifampicin. pTF2 has a transfer frequency of 3×10^{-4} after one hour of conjugation to *E. coli* J53-2 [9]. However, if using other media, strains, or plasmids, it is required to identify the optimal time of conjugation. Keep the conjugation time as short as possible, to avoid growth during conjugation, as donor and recipient may have different growth rates.

Luria-Bertani (LB) broth (Invitrogen, Barcelona, Spain) was used for bacterial growth. Media was supplemented with antibiotics when needed (Sigma, Copenhagen, Denmark) including 2 μg/mL cefotaxime (CTX) and 50 μg/mL rifampicin (Rif). Bacterial strains were grown at 37 ˚C.

Compound library and plate controls

A set of 3500 chemically synthesized compounds were provided by ABAC Therapeutics, Barcelona, Spain, in DMSO stocks at a concentration of 12,8 mg/mL. The concentration used in this experiment corresponds to the highest possible (1 % DMSO to culture volume), without the DMSO having a killing effect on bacterial strains.

In addition, the following controls were included, 1) a control of conjugation consisting of donor + recipient, 2) control of inhibition consisting of donor + recipient + benzyl isothiocyanate (32 μg/mL), 3) a donor control to confirm the donor's sensitivity to Rif, 4) a recipient control to confirm the recipient's sensitivity to CTX and 5) a negative control of LB broth alone to detect any media contamination.

The control compound benzyl isothiocyanate, has previously been shown to inhibit conjugation [10]. A concentration of 32 μg/mL was shown to have an inhibitory effect on plasmid conjugation under our conditions.

Compound screening protocol

The screening assay was performed in 96-well round bottom plates. Each plate was loaded with 88 compounds to a final concentration of 128 μg/mL.

Bacterial cultures of donor and recipient were prepared by diluting an overnight culture to OD_{600}=0.05 in LB and grown to OD_{600}=0.5 (mid exponential phase) at 37 ˚C. Then 100 uL of recipient was added to the 88 wells containing compound, followed by the addition of 100 uL donor to the same wells. Furthermore, the 5 controls were added to the remaining empty wells, with conjugation control (control 1) added to two wells and LB control (control 5) being loaded in three wells. Some of the three LB controls can be replaced with either other controls or compounds if needed. The 96-well plates were then placed in the incubator at 37 ˚C for an hour (conjugation time needs to be adjusted based on the plasmid and strains used) 10 μL from each well were then spotted as single drops on LB agar plates supplemented with 2 μg/mL CTX and 50 μg/mL Rif using a multichannel pipette to select for transconjugants. Plates were incubated at 37 ˚C overnight.

Control 1 should reveal growth, a sign of conjugation. Controls 2, 3, 4, and 5 should be without visible growth on the agar plate. The effect of the compounds was evaluated visually by eye, by comparing the spots to the controls (Fig. 1). Compounds that showed no colonies or a highly reduced number of colonies (single colonies could be observed when compared to control 1) were re-tested under the same conditions to confirm their effect. Ensure to re-test the compounds at a different location in the microtiter plates and on the agar-plate compared to the first round, to ensure that results are not biased due to technical reasons like variations in microtiter plates/agar plates or position in incubator.

Secondary confirmatory assays

Additional methods can be used to further evaluate the compounds. As compounds may affect bacterial division, and thereby potentially affect conjugation, microscopic analysis of donor and recipient grown with or without compounds, could be included. In addition, it can be investigated whether the compounds have an effect on bacterial growth either using manual measurements or using an automated microbiology growth curve analysis system like Bioscreen C^{TM} (Oy Growth Curves Ab Ltd, Finland) and compare bacterial growth patterns between bacteria grown with and without the individual compounds. Furthermore, colony-forming unit (CFU) counts could be performed alongside optical density (OD) measurements to ensure that OD is reflecting the number of viable bacterial cells in culture. Confirmation of conjugation inhibition should also be done; in case the screening set-up does not completely reflect normally used conjugation protocol. It is recommended to use conditions (media, temperature and starting inoculum) that mimic the conjugation experiment conditions when performing additional assays.

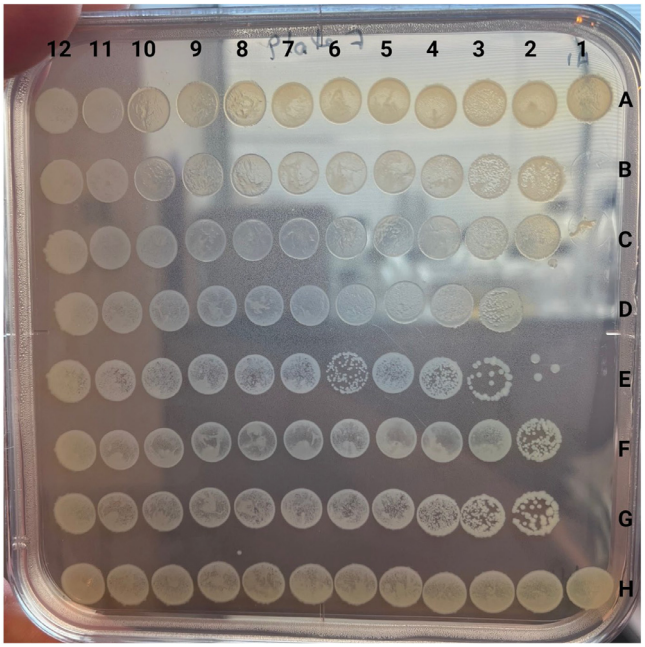

Fig. 1. Representative result plate from the compound library screen used to detect inhibitory compounds of *E. coli* plasmid conjugation. Lane 2 to 12 shows the level of conjugation when donor and recipient are exposed to individual compounds. Lane 1 represents the controls: Spot A1 and H1 consists of donor + recipient, spot B1 consists of donor + recipient + benzyl isothiocyanate (32 μg/mL), spot C1 consists of donor alone, spot D1 consists of recipient alone and spots E1, F1 and G1 consist of LB broth alone. Spot D2 shows a compound that fully inhibits conjugation and spots E2, E3, E6, F2, G2 and G3 show compounds that reduce conjugation compared to the control spots A1 and H1.

Method validation

The first round of screening showed that 62 out of 3500 compounds (1.77 %) had an effect on the conjugation of plasmid pTF2 from *E. coli* MG1655 into *E. coli* J53–2. 40 reduced conjugation which was shown as a reduced number of colonies compared to conjugation control, and 22 inhibited conjugation, which was shown as a spot without any growth. These compounds were retested using the same method to validate their effect and 10 out of the 62 (16.1 %) showed the same result after the second round (two reduced conjugation and eight inhibited conjugation). The reduction in positive hits from round 1 to round 2 can be explained by natural fluctuations in conjugation frequencies, meaning that increases in conjugation rate reduces number of spots with single colonies. False positives may also be caused by experimental factors, as the compounds were placed at different positions in the wells of the microtiter plates, and variations in plates, positions in incubator or experimental errors may have affected the outcome.

For the compounds that affected conjugation, we wanted to identify the compounds with anti-conjugation activity and discard compounds with antibacterial activity. Therefore, the donor and recipient were grown separately in LB broth without and with 128 μg/mL of compound. In 4 out of 10 compounds (40 %), the donor and the recipient strains did not grow (clear media with no growth after 18–24 h of growth), and these compounds were not further tested. Therefore, six compounds in total were qualified for further testing, two that reduced conjugation and four that fully inhibited conjugation.

These results show that this method is a fast and easy way to identify compounds that have an effect on conjugation from a large compound library without the need for special equipment. In addition, another advantage of this screening method is that it can be applied to any conjugative plasmid from any bacterial strain. Furthermore, the protocol may also be used to screen for compounds inhibiting conjugative transfer of other mobile genetic elements.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Domingo Gargallo-Viola is CEO at ABAC Therapeutics S.L. The company works on finding new pathogen-specific compounds with novel mechanisms of action. Domingo Gargallo-Viola has provided the compound library used for setting up the method described in this paper.

CRediT authorship contribution statement

Jennifer Moussa: Conceptualization, Methodology, Writing – original draft. **Domingo Gargallo-Viola:** Conceptualization, Supervision, Writing – review & editing. **Line Elnif Thomsen:** Conceptualization, Supervision, Writing – review & editing.

Data availability

The data that has been used is confidential.

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