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Reprogramming Targeted-Antibacterial-Plasmids (TAPs) to achieve broad-host range antibacterial activity

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ABSTRACT

The emergence and spread of antimicrobial resistance results in antibiotic inefficiency against multidrug resistant bacterial strains. Alternative treatment to antibiotics must be investigated to fight bacterial infections and limit this global public health problem. We recently developed an innovative strategy based on mobilizable Targeted-Antibacterial-Plasmids (TAPs) that deliver CRISPR/Cas systems with strain-specific antibacterial activity, using the F plasmid conjugation machinery for transfer into the targeted strains. These TAPs were shown to specifically kill a variety of Enterobacteriaceae strains, including *E. coli* K12 and the pathogen strains EPEC, *Enterobacter cloacae* and *Citrobacter rodentium*. Here, we extend the host-range of TAPs using the RP4 plasmid conjugation system for their mobilization, thus allowing the targeting of *E. coli* but also phylogenetically distant species, including *Salmonella enterica* Thyphimurium, *Klebsiella pneumoniae*, *Vibrio cholerae*, and *Pseudomonas aeruginosa*. This work demonstrates the versatility of the TAP strategy and represents a significant step toward the development of non-antibiotic strain-specific antimicrobial treatments.

1. Introduction

The emergence and extensive spread of multidrug resistant bacterial pathogens is a major health problem that requires the development of innovative antibacterial treatments. Many strategies alternative to antibiotics have been developed over the last few years, notably the use of bacterial conjugation as a mechanism to deliver DNA with antibacterial activities. While some strategies rely on the transfer of toxins (Collins et al., 2019; López-Igual et al., 2019; Shankar et al., 2007; Starčič Erjavec et al., 2015), others use antimicrobials based on Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the associated Cas proteins (Dong et al., 2019; Hamilton et al., 2019; Hao et al., 2020; Ji et al., 2014; Neil et al., 2021; Rodrigues et al., 2019; Ruotsalainen et al., 2019; Wang et al., 2019; Wongpayak et al., 2021). CRISPR/Cas systems are programmable and highly specific as they rely on the recognition of ~20 nucleotides sequences by a guide RNA (gRNA) that recruits the Cas9 protein to the complementary targeted DNA (Anders et al., 2014), with the ability to discriminate target sequences that differ by a single nucleotide mutation (Citorik et al., 2014; Semenova et al., 2011).

To invest efforts in the research of alternative strategies, we have recently developed an innovative antimicrobial approach that uses conjugation to deliver CRISPR systems exerting an antibacterial activity on specifically targeted pathogenic strains (Reuter et al., 2021). This method relies on synthetic Targeted-Antibacterial Plasmids (TAPs) carrying an origin of transfer $oriT_{helper}$ recognized by the relaxosome and mobilized *via* the type IV secretion system (T4SS) produced in *trans* by a helper conjugative plasmid contained in the donor cells (Fig. 1). Once transferred and established in the recipient cells, TAPs produce a CRISPR system that induces double-strand DNA breaks (DSBs) on chromosomal or plasmid DNA by the Cas9 nuclease (Fig. 1 (i)) (Cui and Bikard, 2016; Gomaa et al., 2014; Jiang et al., 2013) or CRISPR interference repressing gene expression by the catalytically dead version dCas9 (Fig. 1 (ii)) (Bikard et al., 2013; Qi et al., 2013). Depending on the induced CRISPR activities and the targeted sequence the targeted strain can then be killed or resensitized to a drug. The initially developed TAPs carry the origin of transfer of the F plasmid to be mobilized by the F plasmid transfer machinery. These TAPs induce efficient and strain-specific antibacterial activity (Reuter et al., 2021), but the range of bacterial species they can target is limited by the narrow host-range of the F plasmid that only transfers among phylogenetically close enterobacteria species. In this work, we modulate the host-range of TAPs by making them mobilizable by the broad host-range RP4 plasmid machinery and demonstrate their ability to induce efficient killing of

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phylogenetically distant γ proteobacteria.

2. Material and methods

2.1. Bacterial strains, plasmids, primer and growth culture conditions

Bacterial strains, plasmids and primers are listed in Tables S1, S2 and S3, respectively. Cells were grown at 37 °C in Luria-Bertani (LB) broth, in M9 medium supplemented with glucose (0.2%) and casamino acid (0.4%) (M9-CASA). When appropriate, supplements were used in the following concentrations; ampicillin (Ap) 100 μg/ml, tetracycline (Tc) 10 μg/ml, kanamycin (Kn) 50 μg/ml except 25 μg/ml for *Vibrio cholerae*, streptomycin (St) 20 μg/ml except 200 μg/ml for *V. cholerae*, and 2 mg/ml for *Pseudomonas aeruginosa*, chloramphenicol (Cm) 20 μg/ml and gentamycin (Gm) 10 μg/ml except 30 μg/ml for *P. aeruginosa*.

2.2. TAPs construction

TAP_{RP4}-Cas9-nsp constructions were done by Gibson Assembly (Gibson et al., 2009) and verified by Sanger sequencing (Eurofins Genomics). To replace the nsp spacer, a new spacer is constructed by annealing 2 oligonucleotides with complementary sequences to the non-cohesive ends generated by *Sap*I restriction of TAP_{RP4}-Cas9-nsp plasmids. Ligation production between the new spacer fragment and the TAP backbone was transformed into *Escherichia coli* DH5α strains. Constructions were verified by PCR reaction and Sanger sequencing (Eurofins Genomics).

2.3. Conjugation assays

2.3.1. Liquid condition

Overnight cultures grown in LB of recipient and donor cells were diluted to an A_{600} of 0.05 and grown further at 160 rpm to reach an A_{600} comprised between 0.7 and 0.9. 25 μl of donor and 75 μl of recipient cultures were mixed into an Eppendorf tube and incubated for 90 min at 37 °C. 1 ml of LB was added gently and the tubes were incubated again for 90 min at 37 °C. Conjugation mixes were vortexed, serial diluted, and plated on LB agar supplemented with antibiotics to select recipients and transconjugants.

2.3.2. Filter condition

Overnight cultures grown in LB of recipient and donor cells were diluted to an A_{600} of 0.05 and grown further at 160 rpm to reach an A_{600} 0.5. 750 μl of donor and 750 μl of recipient cultures were centrifuged 10 min at 5000 rpm. The pellet was resuspended into 50 μl of LB and then dropped on a filter (MF Membrane Filters- 0,45 μm HA- Ref HAWP02500 Millipore) placed on LB agar medium. Conjugation mix were incubated for 2 h at 37 °C, then resuspended into 1 ml of LB, serial diluted, and plated on LB agar supplemented with antibiotics to select recipients and transconjugants.

2.4. pSLT detection

Detection of pSLT plasmid region of interest was performed by PCR (Polymerase Chain Reaction) with primers listed in Table S3. The sequence of pSLT was verified by sequencing PCR products (Eurofins Genomics).

2.5. Live-cell microscopy imaging and analysis

2.5.1. Time-lapse experiments

Overnight cultures grown in M9-CASA of recipient and donor cells were diluted to an A_{600} of 0.05 and grown further at 160 rpm to reach an A_{600} 0.5. 25 μl of donor and 250 μl of recipient cultures were mixed, gently vortexed and loaded to B04A microfluidic chamber (ONIX, CellASIC®) with 5 psi for 1 min. Nutrient supply was maintained at 1 psi and the temperature maintained at 37 °C. Cells were imaged every 10 min for 3 h.

2.5.2. Snapshot imaging

Overnight cultures grown in M9-CASA of non-fluorescent *E. coli*, mRuby producing *E. coli* and TAP_{RP4}-Cas9-mRub escape mutants were diluted to an A_{600} of 0.05 and grown further at 160 rpm to reach an A_{600} 0.5. 10 μl samples of culture were spotted onto an M9-CASA 1% agarose pad on a slide (Lesterlin and Duabrry, 2016) and imaged directly.

2.5.3. Image acquisition

Conventional wide-field fluorescence microscopy imaging was carried out on an Eclipse Ti-E microscope (Nikon), equipped with x100/1.45 oil Plan Apo Lambda phase objective, FLash4 V2 CMOS camera (Hamamatsu), and using NIS software for image acquisition. Acquisitions were performed using 50% power of a Fluo LED Spectra X light source at 488 nm and 560 nm excitation wavelengths. Exposure settings were 100 ms for sfGFP and 100 ms for mRuby2.

2.5.4. Image analysis

Quantitative image analysis was done using Fiji software with MicrobeJ plugin (Ducret et al., 2016). The Manual-editing interface of MicrobeJ was used to optimize cell detection and the mean intensity fluorescence and cell length parameters were automatically extracted

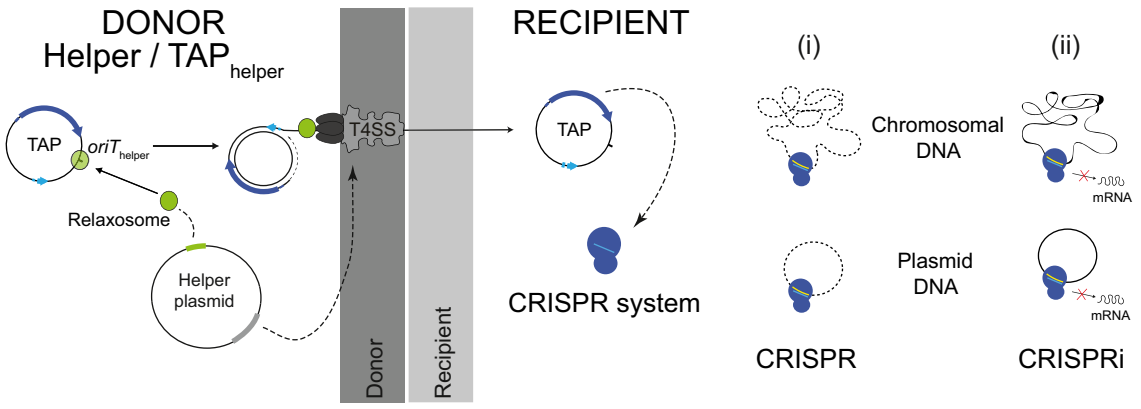


Fig. 1. Diagram of the TAP antibacterial strategy. A donor strain produces the helper plasmid conjugation machinery, which consists of the relaxosome recognizing the origin of transfer of the helper plasmid ($oriT_{helper}$) carried by the TAP_{helper}, and the T4SS transferring the TAP_{helper} DNA to the recipient cells. Once established, the TAP produces a CRISPR system that recognizes a targeted sequence (yellow line) to induce double-strand DNA breaks (i) or gene expression inhibition (ii). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and plotted. We defined the timing of TAP acquisition (time $t = 0$) by analyzing the increase of the sfGFP fluorescence signal conferred by the TAPs. Plasmid acquisition was validated when a 12% sfGFP fluorescence increase was observed in the transconjugant cells. Fluorescence profiles of each cells were then aligned according the defined t = 0 to generate the graphs presented in Fig. 3D and E.

3. Results

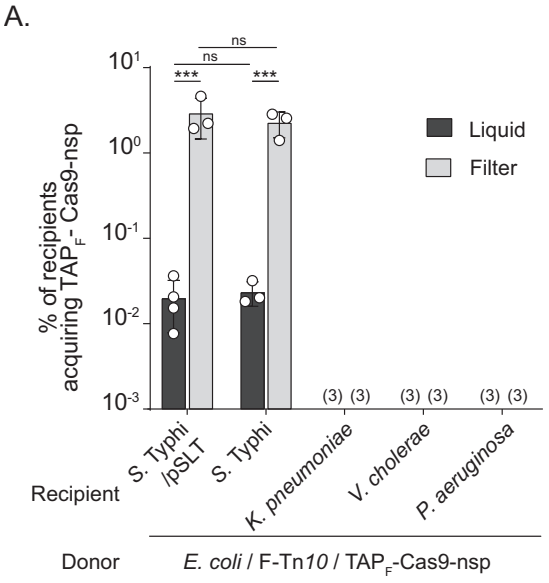
3.1. The F plasmid conjugation system transfers TAP inefficiently to phylogenetically distant species

TAP_F are efficiently transferred by the F-Tn*10* conjugation machinery in *E. coli*, *C. rodentium* and *E. cloacae* (Reuter et al., 2021). We first examined the transfer efficiency of a TAP_F-Cas9-nsp coding for the Cas9 and a non-specific (nsp) crRNA spacer that does not target any sequence of the recipient species, using an *E. coli* donor and a larger set of recipient strains. In liquid mating conditions, ~0.02% of *Salmonella enterica* Thyphimurium strain LT2 cells acquire the plasmid, while no transfer was detected toward the phylogenetically distant species *Klebsiella pneumoniae* strain LM21, *Vibrio cholerae* O1 biovar El Tor str. N16961 and *Pseudomonas aeruginosa* strain PAO1 (Fig. 2A). Since the TAP_F carry the broad-host range pBBR1 origin of replication, active in those strains (Szpirer et al., 2001), these discrepancies can be attributed to the variability of transfer efficiency. Transfer DNA efficiency can vary between liquid and solid media mating conditions (Bradley et al., 1980). This is usually attributed to the ability to establish stable contact between donor and recipient cells. Results show that conjugation on solid filter media does not improve TAP_F-Cas9-nsp transfer into *K. pneumoniae*, *V. cholerae* and *P. aeruginosa* recipient strains (Fig. 2A). However, we observe a ~ 2 log increase in the transfer efficiency in *S.* Thyphimurium recipient cells, likely due to the facilitated establishment of stable mating pairs with an *E. coli* donor. Of note, the *S. enterica* Thyphimurium strain LT2 also contains the IncF-like pSLT plasmid (Fig. 2B) that could interfere with the mobilization of TAP_F by the F plasmid conjugation machinery through exclusion mechanism mediated by surface and entry exclusion proteins TraT and TraS respectively (Garcillán-Barcia and de la Cruz, 2008). To test this hypothesis, we performed conjugation with pSLT-free *S.* Thyphimurium recipient cells (Fig. 2B) and observed equivalent TAP_F-Cas9-nsp transfer frequency (Fig. 2A) showing that the pSLT plasmid does not mediate exclusion of the F plasmid. This is consistent with the fact that exclusion is mainly due to the activity of TraS entry exclusion proteins (Achtman et al., 1977), and that $TraS_F$ and $TraS_{pSLT}$ have only 12% amino acid sequence conservation. $TraT_F$ and $TraT_{pSLT}$ share 90% amino acid identity but TraT surface exclusion proteins are known to play a minor role in exclusion (Garcillán-Barcia and de la Cruz, 2008).

3.2. Validation of CRISPR activity of TAP_{RP4} mobilized by the RP4 plasmid conjugation machinery

In order to broaden TAPs' host spectrum and target phylogenetically distant species, we developed a conjugation system based on the broad-host range IncP conjugative RP4 plasmid. Although the conjugation frequency of RP4 plasmid is low in liquid condition and reaches a maximum of 0.1% after 3 h of mating, almost 100% of *E. coli* recipient cell population can acquire RP4 plasmid after 2 h of mating on solid filter (Supplementary Fig. 1). This makes it a promising tool for testing in TAPs mobilization. Therefore, we constructed a new set of TAPs carrying the $oriT_{RP4}$ of the RP4 plasmid (instead of the $oriT_F$ of the F plasmid) to render them mobilizable by the RP4 helper plasmid conjugation machinery (Fig. 3A). The TAP_{RP4} derivatives, like the original TAP_F, carry the broad host-range pBBR1 origin of replication, a selection of resistant gene cassettes, the CRISPR system composed of the *Streptococcus pyogenes* wild-type *cas9* or the catalytically dead variant *dcas9* genes, and the guide gRNA. Optionally, the *superfolder green fluorescent protein* (*sfgfp*) or *mcherry* genes expressed from broad-host range synthetic Biofab or tac promoters can been added for monitoring transfer in live-cells under the microscope (Reuter et al., 2021) (Fig. 3A). TAP_{RP4}-Cas9-nsp plasmid, is efficiently mobilized by the RP4 conjugation machinery as ~85% of an *E. coli* recipient cell population has received the plasmid after 2 h of mating on filter (Fig. 3B). Next, TAP_{RP4} ability to induce antibacterial activity was validated by using the mRub spacer that targets the *mruby2* gene inserted into the chromosome of the *E. coli* recipient cells (Vidakovic et al., 2018). We observed a ~ 4.5 log loss in the viability of TAP_{RP4}-Cas9-mRub transconjugants compared to TAP_{RP4}-Cas9-nsp transconjugants (Fig. 3B), confirming that Cas9 targeting and inducing a DSB at a single chromosome locus is lethal for the strain.

Using live-cell microscopy, we analyzed the effect of the TAP_{RP4} acquisition at the single cell level. In this experiment, TAP_{RP4} carry the sfGFP reporter enabling green fluorescence to be produced in donor and transconjugant cells. The recipient cells produce the mRuby reporter that confers red fluorescence (Fig. 3C). Monitoring the effect of TAP_{RP4}



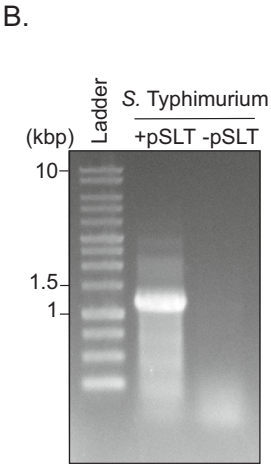
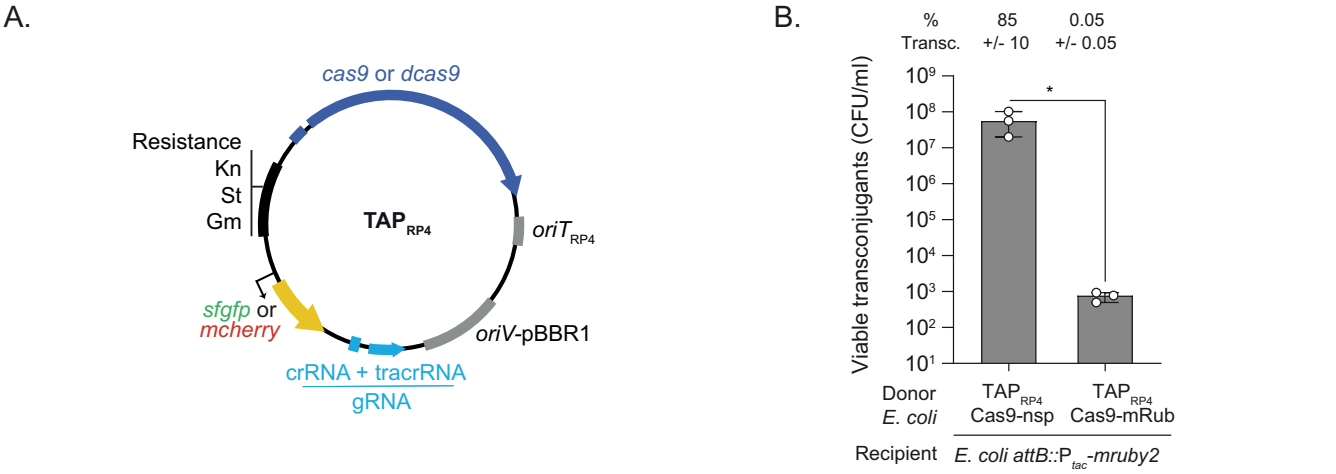
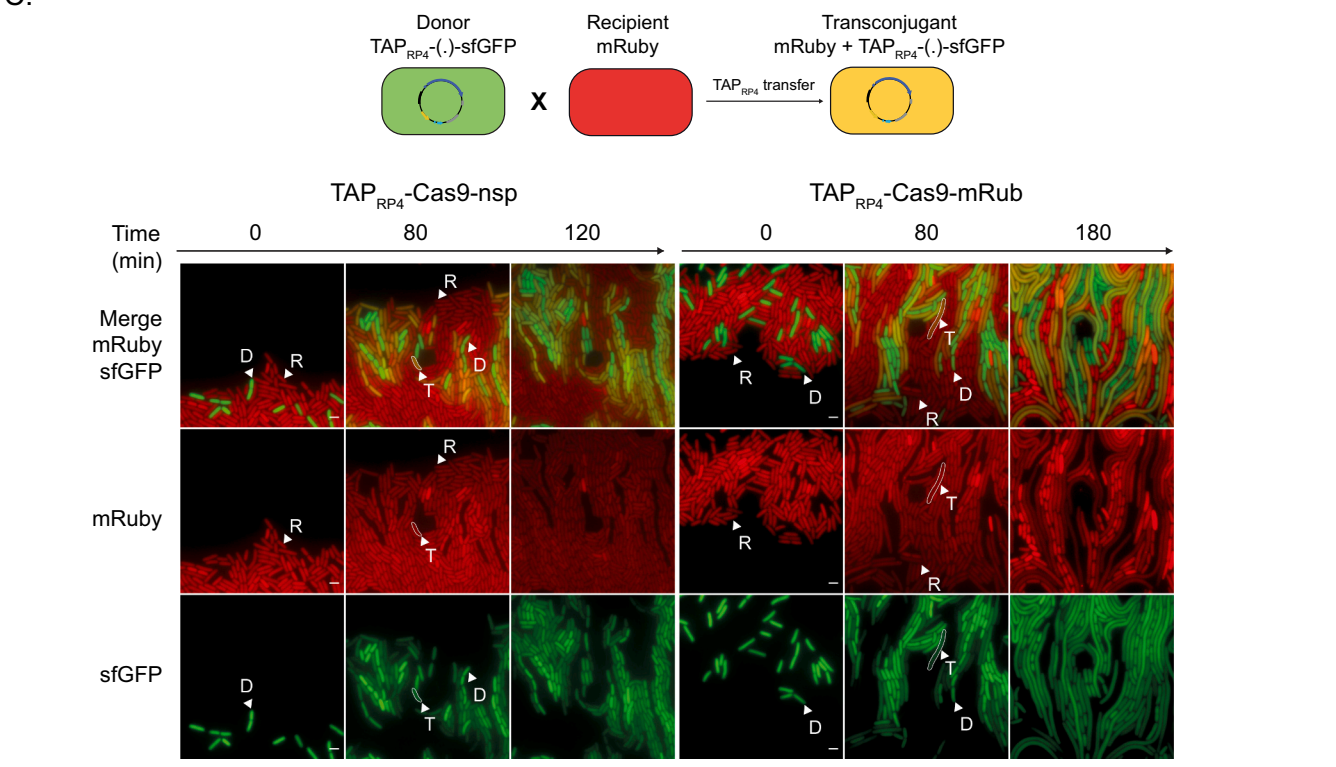


Fig. 2. The transfer of TAP_F by the F plasmid conjugation machinery is inefficient in *K. pneumoniae*, *V. cholerae* and *P. aeruginosa*. (A) Histograms of TAP_F-Cas9-nsp transfer estimated by plating assays after 3 h or 2 h of mating in liquid or filter condition respectively. Donor *E. coli* TAP_F-Kn-Cas9-nsp (LY1369) or *E. coli* TAP_F-St-Cas9-nsp (LY2459); recipients *S.* Typhimirium carrying the pSLT plasmid (LY1977), pSLT plasmid free *S.* Typhimirium (LY2480), *K. pneumoniae* (LY1182), *V. cholerae* (LY1706) and *P. aeruginosa* (SB97). Number in brackets indicate replicates with a detection limit of transconjugants below 10^{-7}. Mean and SD are calculated from at least three independent experiments. *P*-value significance *** (<0.009) and ns (>0.05 non-significant) were obtained from multiple *t*-tests and Mann-Whitney test respectively. (B) Analysis by PCR of the presence (+pSLT) and absence (-pSLT) of the pSLT plasmid in *S.* Thyphimurium LY1977 and LY2379 strain respectively. Ladder in kbp is shown on the left.





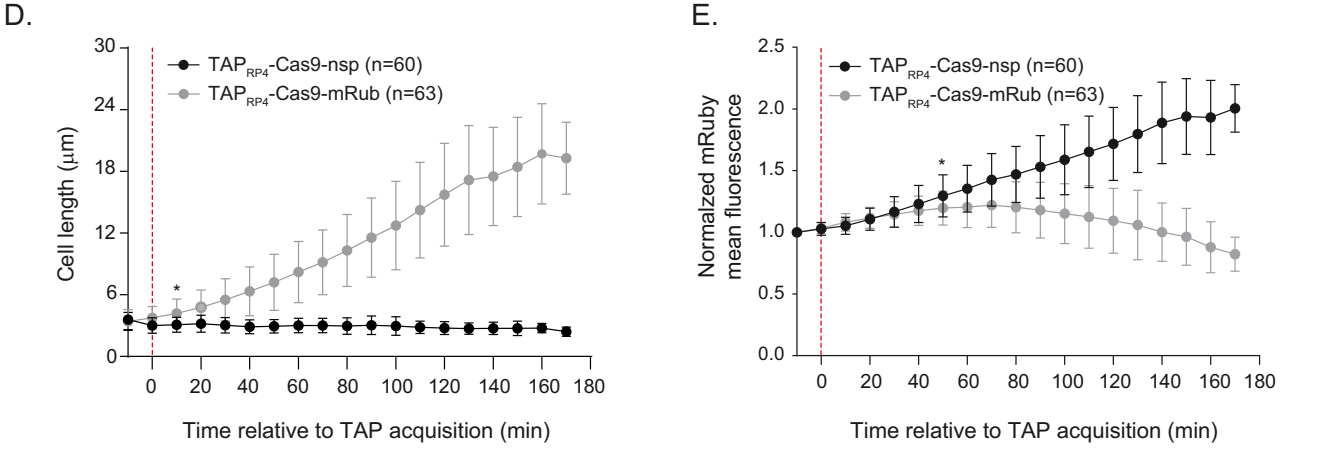


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Fig. 3. Transfer of TAP_{RP4} by the RP4 machinery mediates killing of a targeted *E. coli* strain. (A) TAP_{RP4} consist of CRISPR system composed of wild-type *cas9* or catalytically dead *cas9* (*dCas9*) genes expressed from the weak constitutive *BBaJ23107* promoter and a gRNA module expressed from the strong constitutive *BBaJ23119* promoter; the RP4 plasmid origin of transfer ($oriT_{RP4}$); the pBBR1 origin of replication (*oriV*), and a set of resistance cassettes (Kn, kanamycin; St, streptomycin; Gm, gentamycin), an optional cassette carrying the *sfgfp* or *mcherry* genes highly expressed from broad-host range synthetic BioFab or tac promoters. (B) Histograms of viable transconjugants after TAP_{RP4} acquisition estimated by plating assays after 2 h of filter mating. Donors *E. coli* TAP_{RP4}-St-Cas9-nsp (LY2211) or TAP_{RP4}-St-Cas9-mRub (LY2212); recipient *E. coli attB*::P_{tac}-*mruby2* (LY1528). Mean and SD are calculated from three independent experiments. *P*-value * significance (<0.02) was obtained from two-tailed unpaired *t*-test. Percentage of transconjugants (ratio T/R + T) are indicated. (C) Real-time visualization of TAP_{RP4} acquisition by *E. coli* recipient cells. Upper panel: diagram of the fluorescent reporter system allowing microscopy visualization of TAP_{RP4} transfer. Donor cells exhibit diffuse green fluorescence due to sfGFP production from TAP_{RP4}; recipient cells exhibit diffuse red fluorescence due to chromosomal mRuby2 production; transconjugant cells are identified by the production of both green and red fluorescence. Lower panel: timelapse microscopy images performed in a microfluidic chamber. D (donor), R (recipient) detoured by a white line, and transconjugant (T) cells are indicated. Scale bar 1 μm. Donors *E. coli* TAP_{RP4}-St-Cas9-nsp-sfGFP (LY2203) or TAP_{RP4}-St-Cas9-mRub-sfGFP (LY2201); recipient *E. coli attB*::P_{tac}-*mruby2* (LY1528). (D-E) Single-cell time-lapse quantification of transconjugant cell length (D) and mRuby mean fluorescence normalized to the −10 min (E). Mean and SD are indicated (n cells analyzed). The time of TAP acquisition (red dashed line at 0 min) corresponds to a 12% increase in green fluorescence in the transconjugant cells. Multiple t-test corrected were performed with Holm-Sidak method. Stars indicate the time with significant difference (*p*-value<0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acquisition by quantifying the apparition of green fluorescence in recipient cells reveals that the acquisition of the TAP_{RP4}-Cas9-mRub triggers cell filamentation reflecting the induction of lethal DSBs (Fig. 3D and movie S1). In addition, we analyzed in transconjugant cells the mRuby mean fluorescence and showed that the acquisition of TAP_{RP4}-Cas9-mRub is followed by a decrease in mRuby intracellular levels (Fig. 3E). By contrast, TAP_{RP4}-Cas9-nsp acquisition has no impact on the cell cycle as the transconjugant exhibit normal cell size and division/proliferation (Fig. 3D and movie S2), and the mRuby production is constant and unaffected (Fig. 3E). Together, these results show that the TAP_{RP4} are efficiently transferred to recipient cells and achieved selective killing of targeted strain.

3.3. TAP_{RP4} escape mutants

We observed the appearance of transconjugants at a frequency of $\sim 10^{-5}$ that are able to survive despite the acquisition of the TAP_{RP4}-Cas9-mRub (Fig. 3B). We previously described these TAP-escape-mutants, which avoid CRISPR activity by either inactivating the CRISPR system directly on TAP or by modifying the targeted locus through point mutations, small or large deletions (Reuter et al., 2021). Nonetheless, we observe a 10-fold decrease in the frequency of TAP_{RP4}-Cas9-mRub escapers (1.25×10^{-5}) in *E. coli* when compared to TAP_F targeting the *lacZ* gene (2×10^{-4}) (Reuter et al., 2021). This could be due to different ability of the considered spacers to bind or cleave the target. Indeed, targeting efficiency can vary by ~2 log from one gRNA to another (Cui and Bikard, 2016; Hamilton et al., 2019). Alternatively, the discrepancy could account for the method used to counter-select the TAP_{RP4}-Cas9-mRub transconjugants. Indeed, transconjugant cells are selected thanks to their kanamycin resistance encoded by the *aph(3′)-IIb* gene, which is located downstream of the targeted *mruby2* gene. As a result, escape mutants that avoid CRISPR activity by making large deletions around the targeted *mruby2* gene might also delete the *aph(3′)-IIb* gene preventing their selection on kanamycin plates. We then hypothesized that the vast majority of selected escape mutants should carry mutations that inactivate the TAPs' CRISPR system. This possibility was confirmed by analyzing the mRuby fluorescence of 10 clones that escaped the TAP_{RP4}-Cas9-mRub activity, which reveals that 9 out 10 escape mutants produce fluorescence at a level comparable to recipient cells indicating that the major escape mechanism did not modify the *mruby2* targeted locus (Supplementary Fig. 2).

3.4. TAP_{RP4} is a potent antibacterial tool to target phylogenetically distant strains

We quantified the efficiency of TAP_{RP4}-Cas9-nsp transfer in phylogenetically distinct strains and compared it to TAP_F-Cas9-nsp in filter condition. The efficiency of TAP_{RP4}-Cas9-nsp transfer to *S.* Thyphimurium carrying or not the pSLT plasmid is comparable to that of TAP_F-Cas9-nsp. However, contrasting with the TAP_F-Cas9-nsp, the TAP_{RP4}-Cas9-nsp is transferred to *K. pneumoniae*, *V. cholerae* and *P. aeruginosa* recipient strains with a frequency varying from 1.1×10^{-2} to 3.5×10^{-1}

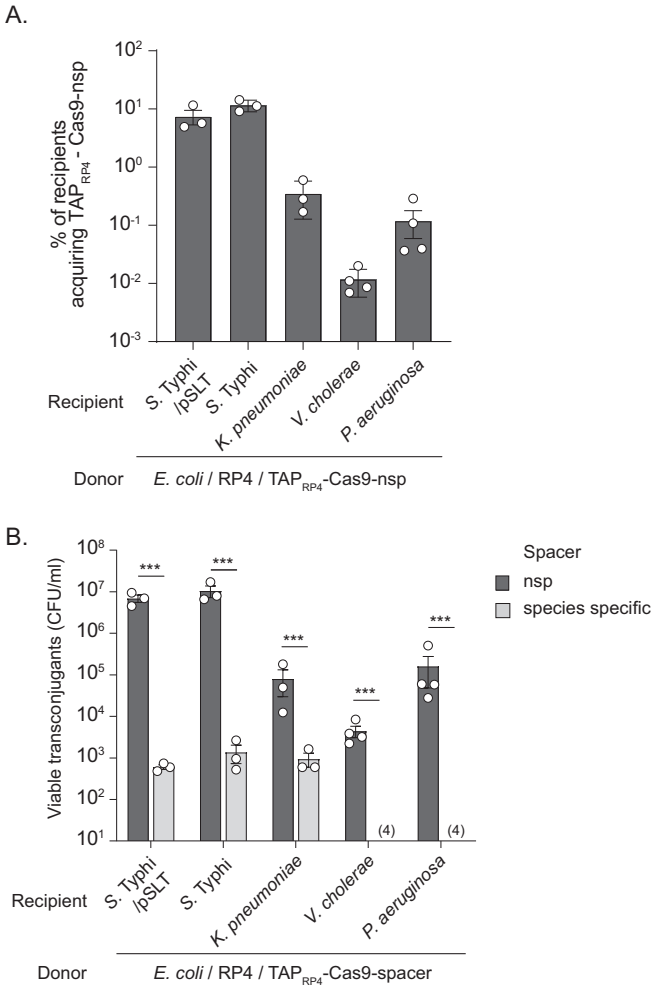

Fig. 4. Efficient phylogenetically distant strain killing by TAP_{RP4}. Histograms of (A) TAP_{RP4}-Cas9-nsp transfer and (B) viable transconjugants estimated by plating assays after 2 h of filter mating. Mean and SD are calculated from at least three independent experiments. Numbers in brackets indicate replicates with a detection limit of transconjugants below 10^{-8}. *P*-value *** significance (<0.009) was obtained from multiple *t*-tests performed on $\log_{10}$ values. Donors *E. coli* TAP_{RP4}-Gm-Cas9-nsp (LY1981), TAP_{RP4}-Gm-Cas9-Sthy (LY2013), TAP_{RP4}-Gm-Cas9-Kp (LY2052), TAP_{RP4}-Kn-Cas9-nsp (LY2386), TAP_{RP4}-Kn-Cas9-Vc (LY2413), TAP_{RP4}-St-Cas9-nsp (LY2067), TAP_{RP4}-St-Cas9-PAO1 (LY2068); recipients *S.* Typhimirium with (LY1977) or without (LY2480) the pSLT plasmid, *K. pneumoniae* (LY1182), *V. cholerae* (LY1706) and *P. aeruginosa* (SB97).

(Fig. 4A). This first confirms that the TAPs' pBBR1 replication origin is indeed functional in these species. The results then show that using the RP4 plasmid conjugation system to broaden the host range of TAPs allows for the targeting of a broader set of phylogenetically distant recipient strains.

We previously developed a "Crispr Search Tool for Bacteria" CSTB algorithm (https://cstb.ibcp.fr/) to identify appropriate spacer sequences to reprogram TAPs against specific recipient species (Reuter et al., 2021). We asked CSTB algorithm to generate spacer sequences targeting *S.* Thyphimurium strain LT2 (Sthy spacer targeting seven loci), *K. pneumoniae* strain LM21 (Kp spacer targeting two loci), *V. cholerae* O1 biovar El Tor str. N16961 (Vc spacer targeting three loci on each chromosome I and II), *P. aeruginosa* strain PAO1 (PAO1 spacer targeting one locus) without targeting any other bacterial genome present in the database. We quantified that acquisition of TAP_{RP4}-Cas9-Sthy induces a ~ 4 log viability loss of *S.* Thyphimurium carrying or not the pSLT plasmid, TAP_{RP4}-Cas9-Kp a ~ 2 log viability loss of *K. pneumoniae* and that Vc and PAO1 spacers induce at least a ~ 3 and 4-log viability loss respectively, as no TAP_{RP4}-Cas9-Vc *V. cholerae* and TAP_{RP4}-Cas9-PAO1 *P. aeruginosa* transconjugants could be detected (Fig. 4B). As a control, we show that the viability of *S.* Thyphimurium, *V. cholerae* and *P. aeruginosa* recipients, which genomes are not targeted by Kp spacer, is not affected by the acquisition of TAP_{RP4}-Cas9-Kp (Supplementary Fig. 3). These results demonstrate that TAP_{RP4} can be easily programmed to impact selectively the viability of genetically distant recipient species.

4. Discussion

Many antibacterial strategies alternative to antibiotics currently in development focus on CRISPR delivery by bacterial conjugation. Several studies successfully use the broad host range RP4 conjugation system to deliver CRISPR that target *E. coli* (Citorik et al., 2014; Dong et al., 2019; Ji et al., 2014; Neil et al., 2021; Ruotsalainen et al., 2019; Wang et al., 2019; Wongpayak et al., 2021) or *S.* Thyphimurium (Hamilton et al., 2019) *in vitro* and *Citrobacter rodentium in vivo* (Neil et al., 2021). Here, TAPs were adapted to be a broad-host range antibacterial able to exert efficient antibacterial activity against a wider range of pathogenic strains. Our findings show that *K. pneumoniae*, *V. cholerae*, and *P. aeruginosa* species can be targeted by replacing the helper F conjugation system by that of the RP4 plasmid. The ability to broaden TAPs' host range represents an advantage over using bacteriophages as CRISPR delivery methodology, which have intrinsically narrow host range. Broad host range TAPs also offers is the possibility to simultaneously eliminate several phylogenetically distant drug-resistant strains present in a given microbial community by targeting a common resistance gene.

Regardless of the helper conjugation system used, TAPs were transferred to *S.* Thyphimurium with the same efficiency. However, mating on solid media significantly increases TAP_F mobilization by the F plasmid conjugation machinery, which is likely related to an increase in mating pair stabilization. Indeed, during F conjugation in liquid conditions the plasmid outer membrane protein TraN exposed at the surface of the donor cells interacts with the outer membrane (OM) receptor OmpA of the *E. coli* recipient cells to stabilize the mating formation, but mating pair stabilization defect observed with *ompA* recipient mutants is overcome when mating is performed on solid media (Havekes and Hoekstra, 1976; Manoil and Rosenbusch, 1982; Morona et al., 1984). Our findings suggest that the F plasmid protein TraN might not fully recognize the OmpA homologue in *S.* Thyphimurium, but solid media may provide a more stable environment for the conjugative machinery to establish and maintain contact with *S.* Thyphimurium recipient cells to deliver the DNA successfully.

The defect in TAP_F transfer to *K. pneumoniae*, *V. cholerae* and *P. aeruginosa* could be due to an inability of the F plasmid conjugation machinery to mediate transfer or to an inability of TAP_F to replicate in these species. The later possibility is ruled out by the observation that TAP_{RP4} carrying the same pBBR1 replication origin replicate in *K. pneumoniae*, *V. cholerae*, and *P. aeruginosa* as transconjugants. Therefore, our results support the view that the inability to transfer the TAP_F to *K. pneumoniae*, *V. cholerae* and *P. aeruginosa* is due to ineffective transfer by the F plasmid conjugation machinery. Recent work reports that TraN encoded by F-like plasmids mediates the conjugation species specificity by cooperating with distinct OM proteins in recipients cells (Low et al., 2022). The F plasmid TraN that recognizes the *E. coli* OM protein OmpA is effective at mediating transfer in *S.* Typhimirium to a lesser extent than in *E. coli*, but ineffective in *K. pneumoniae* which correlates with the increasing phylogenetic distance between OmpA receptors from *E. coli* to *S.* thyphimurium and *K. pneumoniae* (Low et al., 2022). These would explain why the F helper plasmid cannot transfer TAP_F in the later species. Low et al. (Low et al., 2022) have not tested the TraN specificity toward *V. cholerae* and *P. aeruginosa*, but *V. cholerae* OmpA share even less homology with *E. coli* OmpA as well the OmpA-like protein OprF in *P. aeruginosa* suggesting that F plasmid TraN would not be able to interact with these homologues to mediate transfer. These observations strongly support that the F plasmid transfer machinery is also ineffective toward these species.

TAPs were successfully modified to target a broader set of recipient species, with TAP_{RP4} transfer efficiencies increased by a factor of $\sim 10^6$ to $\sim 10^4$ fold in *K. pneumoniae*, *V. cholerae* and *P. aeruginosa* when compared to TAP_F. However, regardless the helper conjugative system, TAPs' transfer efficiency remains a limiting parameter, being almost 100% between *E. coli* but still varying from 0.01 to 10% between phylogenetically distant donor and recipient strains. This is consistent with the observation that the IncF- and IncP-like plasmids transfer efficiency toward kin is 10-fold higher than toward non-kin (Dimitriu et al., 2019; Dimitriu et al., 2016). Increasing the efficiency of conjugation would require the use of superspreader helper plasmid mutants that can be isolated through random mutagenesis methods, such as Tn-seq approach (Hancock et al., 2020; Yamaichi et al., 2015) or Accelerating Laboratory Evolution (ALE) procedure (Neil et al., 2021). In addition, the investigation of TAPs efficacy *in situ* would be an essential next step in expanding the use of TAPs' strategy, as conjugation efficiency levels vary between *in vitro* and *in situ* conditions (Neil et al., 2020). For example, the IncI1 TP114 plasmid is currently the most promising for antibacterial transfer *in vivo*, even though testing of TP114's transfer ability has been limited to *E. coli* and *C. rodentium* (Neil et al., 2021).

The TAPs design benefits from being versatile and easily engineered. Future research will be required to develop custom TAPs composed of different origin of transfer modules associated with a set of helper plasmids. The creation of such a toolbox would enable the selection of the best-suited TAP system based on the strain(s) to be targeted.

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Author contributions

SD, AR, ED and SB: performed experiments and analyzed data. CL and SB: Funding acquisition, conceptualization. SB: supervision, writing original draft, review & editing.

Declaration of Competing Interest

None.

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