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Engineered toxin-intein antimicrobials can selectively target and kill antibiotic-resistant bacteria in mixed populations

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Targeted killing of pathogenic bacteria without harming beneficial members of host microbiota holds promise as a strategy to cure disease, and limit both antimicrobial-related dysbiosis and development of antimicrobial resistance. We engineer toxins that are split by inteins and deliver them by conjugation into a mixed population of bacteria. Our toxin-intein antimicrobial is only activated in bacteria that harbor specific transcription factors. We apply our antimicrobial to specifically target and kill antibiotic resistant *Vibrio cholerae* in complex populations gathering various bacterial species. We found that 100% of antibiotic resistant *V. cholerae* receiving the plasmid were killed. Escape mutants were extremely rare (10-6-10-8). We demonstrate that conjugation and specific killing of targeted bacteria is functional in the microbiota of zebrafish and crustacean larvae, which are natural hosts for *Vibrio* spp. Toxins split with inteins could form the basis of a range of precision antimicrobials which would kill both Gram – and Gram + pathogens.

With the advent of the antibiotic era, infectious diseases were thought to be under control, but worldwide emergence of antibiotic-resistant bacteria has occurred, owing to the widespread unchecked use of antibiotics. Further, it is now estimated that antibiotic resistant

bacteria could be the main cause of death by 2050[1] unless new classes of antimicrobials are developed.

Broad spectrum antimicrobials indiscriminately kill bacteria which can result in microbiota dysbiosis and concomitant health sequelae. Moreover, antibiotic that have non-specific targets can select for antibiotic resistance, which is mainly acquired by horizontal gene transfer among bacteria in communities[2]. Alternatives to broad spectrum antibiotics include bacteriocins, which kill a subset of bacterial species or strains, and will not provoke a superinfection[3]. Other targeted antimicrobials have also been reported, including CRISPR-Cas antimicrobials[4–6], phage therapy[7] and local release of toxins[8].

We set out to design antimicrobials to specifically kill antibiotic-resistant *Vibrio cholerae*. To mediate bacterial killing we chose the toxin component of type II bacterial toxin-antitoxin (TA) systems, which are involved in stabilization of plasmids, prophages and superintegrons[9]. Type II toxin and antitoxins are proteins[9]. The toxin targets conserved bacterial cellular functions which reduces the potential for development of resistance. Each antitoxin is highly specific for the cognate toxin, and nonspecific toxin-antitoxin interactions are counterselected[10]. Our antimicrobial design relies on the regulation of type II TA transcription by highly specific transcription factors (TF). This means that activation of the toxin, and concomitant killing, of individual members of mixed bacterial populations is feasible if a targeted bacterial species expresses the Type II toxin-regulating transcription

58 factor. We validated our approach by showing that we could selectively kill antibiotic-
59 resistant *V. cholerae* present in mixed populations.

60 *V. cholerae* causes between 21 000 and 143 000 deaths from cholera per year[11].
61 The most recent cholera pandemics involved the O1 and O139 serogroups. Virulence in *V.*
62 *cholerae* is coordinated by the master transcriptional activator ToxR, which regulates the
63 ToxR regulon[12], which includes the cholera toxin genes. Cholera epidemics are associated
64 with antibiotic resistance owing to resistance genes present on an integrative and
65 conjugative element (ICE) named SXT (from sulfamethoxazole and trimethoprim resistance).
66 SXT can carry genes that confer resistance to sulfamethoxazole (*sul*2), trimethoprim (*dfrA*1
67 and *dfr*18), streptomycin (*strB*), chloramphenicol (*floR*) and tetracycline (*tetA*) and was first
68 described in *V. cholerae* serogroup O139[13]. SXT also encodes functions promoting its
69 excision, dissemination by conjugation, and integration, as well as the transcription factors
70 that control expression of these functions[13].

71 Our previous experience with type II toxins[14,15] taught us that basal expression of a
72 full-length toxin gene from P_{BAD} is sufficient to kill the *E. coli* host. To avoid this, we
73 designed a genetic module containing a toxin split by an intein, and in our module the split
74 toxin-intein can only be activated by ToxR. Inteins are protein sequences embedded into a
75 host protein (extein) from which they are autocatalytically excised in a process called protein
76 splicing. During protein splicing the intein ligates the extein extremities and allows the

77 reconstitution of the mature protein. In nature, a few examples of split inteins also exist
78 allowing the assembly of a single protein from two genes[16]. We split the type II toxin gene
79 *ccdB* (Plasmid pToxInt, Supplementary Fig. 1) into two parts, each of which is associated
80 with half of a split intein. Split inteins have been used in several biotechnological tools[17] and
81 enable control of toxic protein functions *in vivo*[18]. We used the split intein DnaE, which is
82 present in the *dnaE* gene of *Nostoc punctiforme*. DnaE is well characterized and has a high
83 rate of trans-splicing[19]. Using inteins enables strict control of toxin production, and avoids
84 toxicity due to basal expression[14,15] (Supplementary Fig. 1).
85 First, we cloned full –length gyrase inhibiting toxin CcdB from *Vibrio fischeri*[15] into a
86 plasmid (pTOX Supplementary Table 1) and transformed the toxin construct into a *E. coli*
87 XL2 blue (Supplementary Table 1) that constitutively expresses a genomic copy of the
88 cognate antitoxin (data not shown). We showed that *ccdB* was bactericidal (Supplementary
89 Fig. 2) and that the intein-mediated splitting strategy led to more stable retention of the toxin-
90 harboring plasmid under repression conditions compared with a construct harboring a whole
91 *ccdB* toxin gene (Supplementary Fig. 1). We also evaluated whether three other type II
92 toxins belonging to different toxin families (ParE2, HigB2 and RelE4[14]) could tolerate a
93 splitting and stay functional, We selected intein insertion points by inspection of 3D structure
94 predictions for toxins made in Phyre2[20], a tool for modeling protein structure (Supplementary
95 Fig. 3a). Each toxin was divided into N- and C-terminal portions (Supplementary Fig. 3b)

96 which were fused in-frame to the N- or C-parts of the split intein *dnaE* gene (102 and 36
97 amino acids long), respectively. N- and C-terminal toxin-intein fusions were cloned in
98 separate, compatible plasmids (N or C plasmids, respectively Supplementary Table 1) and
99 were under the control of different promoters (Fig. 1a). We validated reconstitution of the
100 active toxin by intein protein splicing in *E. coli* (Supplementary Fig. 4). For all five tested split
101 toxins, we found that under inducing conditions bacteria containing N and C plasmids died,
102 whereas bacteria with either the N or the C plasmid survived. N and C toxin-intein complex
103 toxicity was tested using mutations known to prevent splicing. When splicing didn't occur,
104 reconstitution of the toxin did not take place, and bacteria survived (Supplementary Fig. 4).
105 Next we chose the gyrase poison CcdB, which is likely the most extensively
106 characterized type II toxin, to design a toxin-intein antimicrobial specific for pathogenic *V.*
107 *cholerae***.** In *V. cholerae* one of the ToxRS-regulated genes encodes a membrane porin,
108 OmpU[21]. We cloned the N fusion of CcdB-intein downstream of the *ompU* promoter
109 (regulated by ToxRS), and the C fusion under P_{BAD} in the same plasmid (pU-BAD,
110 Supplementary Fig. 5a). The functionality of pU-BAD was tested in an *E. coli* DH5α strain
111 expressing the *V. cholerae toxRS* operon from a second plasmid (pRS, Supplementary Fig.
112 5a). Upon arabinose-mediated induction of *toxRS* expression, only bacteria containing both
113 pU-BAD and pRS plasmids died (Supplementary Fig. 5b). We replicated cell killing in
114 MG1655 (data not shown). We then tested pU-BAD activity in pathogenic *V. cholerae* strains

O1 and O139 (Supplementary Fig. 6a). We observed constitutive expression of the N-fusion due to the presence of chromosomal *toxRS*. However, toxicity due to basal expression from P_{BAD} (Supplementary Fig. 6a) led to pU-BAD plasmid instability in *V. cholerae*. A *V. cholerae* mutant lacking *toxRS* (Δ*toxRS*) displayed normal growth and pU-BAD stability in the presence of arabinose (Supplementary Fig. 6a). This suggested that P_{ompU} could be used to regulate CcdB-intein fusion expression for targeted killing of *V. cholerae*.

In order to develop a conjugative CcdB-intein-based antimicrobial to specifically kill pathogenic *V. cholerae* in microbial communities, we cloned a split-toxin-intein operon under the control of *ompU* promoter in a plasmid, and added an origin of transfer (*oriT*) to render it conjugative (plasmid pPW, Supplementary Fig. 6b, Supplementary Table 1). Conjugation is carried out from donor strain *E. coli* β3914, an MG1655 Δ*dapA* which contains the RP4 conjugative machinery integrated into its chromosome. pPW was introduced by conjugation into *V. cholerae* strains O1, O139 and an O1-Δ*toxRS* mutant (Supplementary Fig. 6b), but only the Δ*toxRS* strain was able to grow after transfer of the pPW plasmid, demonstrating that it kills only *Vibrio* expressing ToxR.

We next tested whether pPW could kill specific strains in a mixed bacterial population (Fig. 1b). Different recipient bacteria in this population could be distinguished in the presence of X-gal: *V. cholerae* O139 (blue) and *E. coli* DH5α (white) (Fig. 1c). We conjugated pPW and two control plasmids (non-toxic N fusion containing pN_{ctrl} plasmid, and

134 the pTox$_{ctrl}$ plasmid, which carries the P$_{BAD}$-regulated toxin-intein operon) into this mixture.
135 After conjugation of pPW from *E. coli* β3914 and selection for transconjugants, pPW killed *V.*
136 *cholerae* O139 (blue bacteria) and we were only able to detect *E. coli* DH5αtransconjugants
137 (white) on media containing XGal. Similarly, after plasmid conjugation into *V. cholerae* O1
138 and *E. coli* strains (MG1655), we only obtained *E. coli* transconjugants (Supplementary Fig.
139 7a).

140 Specific killing by pPW relies on expression of the regulator *toxR,* which is present in
141 all *Vibrio* genera[22]. However, the ToxR regulon has evolutionarily diverged among the
142 different *Vibrio* species, so we analyzed pPW action in two other *toxRS*-containing *Vibrio*
143 species (Fig. 1d). We found that pPW can kill *Vibrio mimicus* but not *Vibrio vulnificus*, which
144 is more phylogenetically distant from *V. cholerae*, and despite harboring a ToxR ortholog,
145 does not activate *ompU* expression[23]. Additionally, we showed that our system is highly
146 specific to ToxR, since conjugation into other γ-proteobacteria, such as *Salmonella*
147 *typhimurium* and *Citrobacter rodentium,* did not result in killing (Supplementary Fig. 7b).

148 Next we evaluated whether a split-intein toxin could kill antibiotic resistant bacteria
149 present in a community. The SXT ICE family in *V. cholerae* includes various antibiotic
150 resistance genes[13]. The SXT chassis encodes several TFs that regulate SXT transmission
151 including the SetR repressor[13]. We designed a module to detect SXT carriage and kill SXT-
152 harboring bacteria by implementing an additional component into our antimicrobial: the *ccdA*

153 gene, which encodes the antitoxin partner of CcdB. *ccdA* was cloned downstream of the
154 SXT PL promoter, which is controlled by the SetR repressor, in a plasmid also containing the
155 *ccdB*-intein operon regulated by the P_{BAD} promoter (pPLA plasmid, Supplementary Fig. 8a,
156 Supplementary Table 1). We tested whether pPLA could kill antibiotic resistant *E. coli* SXT
157 (Supplementary Fig. 8b) and *V. cholerae* O139 (Fig. 2a). Both bacteria contain an SXT
158 element integrated at *prfC*. Only SXT carrying bacteria from both species were killed. All
159 bacteria lacking SXT, including *V. cholerae* O1 and *E. coli* DH5α, survived (Fig. 3a and
160 Supplementary Fig. 8b). In order to develop a conjugative antimicrobial to kill antibiotic
161 resistant bacteria we added an *oriT* to pPLA to produce pABRW (Supplementary Table 1,
162 Fig. 2). pABRW was tested by conjugation into a mixed population of *E. coli* MG1655 (blue)
163 and *E. coli* SXT (white). Selection for pABRW yielded only *E. coli* MG1655 transconjugants,
164 demonstrating that pABRW specifically kills bacteria containing SXT (Fig. 2b). The same
165 result was obtained after conjugation of pABRW into *V. cholerae* O139 mixed with *V.*
166 *cholerae* O1-Δ*lacZ* (Fig. 2c), confirming that pABRW plasmid specifically kills ABR bacteria
167 in a heterogeneous population.

168 We next combined the pPW and pABRW modules in a single plasmid. We replaced
169 the operator sequence O4 of PL with O1 (see online Methods) to increase SetR repression
170 to yield pFW (Supplementary Table 1, Fig. 3), which efficiently kills *V. cholerae* O139 (Fig.
171 3c). In order to test whether non-replicative-conjugative plasmids (which would not spread

toxin-intein fusions and/or antibiotic resistant genes) could harbor our killing module, we changed the pSC101 replication origin to a *pir*-dependent R6K origin (Supplementary Fig. 9). R6K origin can be activated in a host expressing an ectopic *pir* gene in the chromosome. After conjugation of pPW-R6K and pFW-R6K into bacteria that lack the *pir* gene, CFU/ml were reduced by 60% compared with controls (Supplementary Fig. 9b). This suggests that even if the plasmid cannot actively replicate once transferred in the targeted bacteria, expression of the toxin is sufficient to kill these bacteria, while the use of such R6K derivatives is limiting the risk of unnecessary propagation of the killing plasmid.

We moved onto evaluate whether our split-intein toxin could target specific bacteria in natural microbiomes. We tested killing of *V. cholerae* O139 in three niches, each of which is a natural habitat for this pathogen[24]: water, tropical zebrafish and a crustacean. We first tested the versatility of *E. coli* β3914 which is auxotrophic for the diaminopimelic acid (DAP) for delivering conjugative plasmid pNctrl, in absence of DAP and found no difference in conjugation rates (Supplementary Table 2). Although conjugation efficiency decreases 300 fold in water, *V. cholerae* transconjugants were obtained with the control plasmid pN_{ctrl} (Supplementary Table 2), while using pFW, no transconjugants were detected (data not shown). These results indicate that in these conditions too when receiving the pFW, *V. cholerae* was killed. These preliminary data suggest that our method using pFW might hold potential in bioremediation of *Vibrio*-contaminated water.

We also tested pNcrtl and pFW using a zebrafish infection model[25] (Supplementary Fig. 10a). Analysis of the microbiota composition using 16S rRNA analysis on 4 days post fertilization zebrafish larvae detected less than 30 different bacterial species, mostly aerobic including several *Aeromonads, Pseudomonads* and *Stenotrophimonads* (JBB and JMG, unpublished). First we tested localization of both *E. coli* and *V. cholerae*, in the gut of zebrafish larvae. We infected four-day-post-fertilization zebrafish larvae with fluorescently tagged *V. cholerae* O1-GFP and *E. coli*-RFP. Fluorescence microscopy revealed co-localization of both *V. cholerae* O1-GFP and *E. coli*-RFP in the digestive tract (Supplementary Fig. 10a). We then tested specific killing in larvae infected with *V. cholerae* O139 (Fig. 4a, Supplementary Fig. 11b,c). The only *V. cholerae* O139 transconjugants obtained were from conjugation with pN_{ctrl} plasmid. No *V. cholerae* O139 transconjugants were obtained using pFW. Therefore, pFW kills *V. cholerae* O139 in zebrafish larvae (Fig. 4a and Supplementary Fig. 11b). We assessed dysbiosis using observation after plating on different media and didn't find any macroscopic change (Supplementary Fig. 11a). We also used a mixture of 1:1 *V. cholerae* O1 and O139 for larval infection and then infected with *E. coli* β3914 (pNctrl) or β3914 (pFW). We detected pN_{ctrl} transconjugants in both O1 and O139 serogroups, but O1 transconjugants only were obtained after conjugation with pFW (Fig. 4a and Supplementary Fig. 11c). Therefore pFW specifically killed the O139 serogroup.

We also tested pFW in the crustacean *Artemia salina* model which is used for fish feeding and commonly found to carry various *Vibrio* species[26] (Fig. 4b and Supplementary Figs. 10b, 12). We detected co-localization of *V. cholerae* O1-GFP and *E. coli*-RFP in the gut of *A. salina* (Supplementary Fig. 10b) and conjugation with pFW plasmid did not provoke visible change in the *A. salina* microbiota, after sampling of the aerobic species on plates (data not shown). Transconjugants of *V. cholerae* O139 were only detected after conjugation with pN_{ctrl}, but not with pFW, showing that pFW kills *V. cholerae* O139 in *A. salina* larvae (Fig. 4b and Supplementary Fig. 12a). We also infected *A. salina* with a 1:1 mix of *V. cholerae* O1 and O139 and detected pN_{ctrl} transconjugants in O1 and O139 serogroups, but only detected O1 pFW transconjugants (Fig. 4b and Supplementary Fig. 12b).

Our split toxin-intein method can be applied to specifically kill selected bacteria subtypes. We anticipate that our system could be fine-tuned to trigger toxin activation in response to various environmental cues[27] including temperature, salt or pH by adding a conditional protein splicing intein[28]. Inteins are functional in eukaryotic cells[29], so toxin-intein combinations might also be developed for targeted killing of tumor cells. The specificity of our system requires identification of a species-specific transcriptional regulator, and such TFs are widespread in pathogenic and antibiotic-resistant bacterial pathogens[30-31]. The Achilles' heel of precision antimicrobials is delivery into complex communities. Antimicrobials

delivered by conjugation e.g. RNA-guided nucleases[5] have reduced targeted bacterial populations by 2- to 3-log even with a ratio of donor:recipient bacteria of 340:1 [5]. In our experiments, using 1:1 ratios were detected a decrease in targeted bacteria (*V. cholerae*) of 10%, which is equivalent to the conjugation rate. We were able to kill ≈ 90-95% of the ABR *E. coli* after the conjugation of pABRW by increasing ratios of donor to recipient to 10:1 (Supplementary Fig. 13). Phage delivery might be useful [5], but phage have other disadvantages[32], including narrow host range and rapid emergence of phage resistance. One advantage of our system compared with others[4,5,33] is that escape mutants are less frequent (below 10^{-6} - 10^{-8}; Supplementary Table 3). Analysis of escape clones (Supplementary Table 4), when targeting ABR bacteria revealed that between 63 and 90% of these clones had lost the SXT element, and were not ABR (Supplementary Table 5). One of the reasons for the lower chance of escape might be that toxin resistance has not been observed. A different synthetic kill switch based on TA systems was also stable due to minimal escape rates *in vivo*[34]. The dual regulatory system in the Final Weapon (Fig. 3a) functions as an AND-logic gate, increasing effectiveness in the control of toxin production, which only happens when both inputs (pathogenicity and ABR) are present. If delivery of mobilizable antimicrobials can be optimized, appearance of resistant bacteria would be rare.

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AUTHOR CONTRIBUTIONS

D.M. and R.L.-I designed the experiments. J.B.-B and R.L.-I designed and performed the *in vivo* experiments. J.B.-B. performed the microscopy experiments and statistic analysis. D.M., R.L.-I and A.R.-P participated in the conception of the project. R.L.-I and D.M. prepared the manuscript and wrote the article with large participation of J.B.-B, J.-M.G. and A.R.-P.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

The data, plasmids and strains generated for this study, that support our findings are available upon request to the corresponding author.

Figure Legends:

Figure 1. Specific killing of pathogenic *V. cholerae* in mixed population of bacteria mediated by toxin-intein strategy. (a) Schematic representation of the active toxin production from plasmids encoding split toxin (red) combined with split intein (blue) inside a bacterium. The first half of the toxin is fused with N-terminal split intein gene (N plasmid) under the control of P_{BAD} promoter and the second half of the toxin is fused with the C-terminal intein gene (C plasmid) and it is controlled by P_{LAC}. Expression of these fusions is activated by the addition of arabinose and IPTG, respectively. Recognition of the protein fusions takes place by the intein module, which carry out the splicing process, which lead to toxin reconstitution, provoking cell death. (b) Mode of action of the genetic weapon spreading through conjugation in mixed population of bacteria and killing of targeted harmful bacteria. (c) Mixed population of *V. cholerae* O139 (blue) and *E. coli* DH5α (white) as recipients for conjugation using β3914 as donor strain containing pN_{ctrl}, $pTox_{ctrl}$ or pPW plasmids. The *ompU* promoter activated specifically by ToxRS from *V. cholerae* is represented by a circled (+) pink-symbol. Transconjugants were selected in MH + Spectinomycin (Sp), X-gal for color development and arabinose for induction of P_{BAD}. (d) Conjugation of pN_{ctrl}, $pTox_{ctrl}$ and pPW plasmid using β3914 as donor strain in *Vibrio mimicus* and *Vibrio vulnificus*. Transconjugants were selected in MH media + Sp and arabinose for induction of P_{BAD}. Pictures are representative of three independent experiments.

Figure 2. Specific killing of antibiotic resistant bacteria (containing SXT). (a) pPLA plasmid that contains CcdB-intein fusion operon under P_{BAD} control expression

392 and *ccdA* antitoxin under PL promoter (symbolized by an orange circled -) which is
393 repressed by SetR. Growth test of *V. cholerae* O1 or O139 containing pPLA plasmid
394 in MH media + Sp and supplemented with glucose (GLU) or arabinose (ARA). (b)
395 Mixed population of *E. coli* MG1655 (blue) and *E. coli* SXT (white) as recipients for
396 conjugation using β3914 as donor strain containing pN_{ctrl}, $pTox_{ctrl}$ or pABRW
397 plasmids. Transconjugants were selected on MH + Sp, X-gal for species
398 identification and arabinose for induction of P_{BAD}. (c) Mixed population of *V. cholerae*
399 O139-SXT (blue) and *V. cholerae* O1 (white) as recipients for conjugation using
400 β3914 as donor strain containing pN_{ctrl}, $pTox_{ctrl}$ or pABRW plasmids as described in
401 b). Pictures are representative of three independent experiments.
402
403
404 **Figure 3. Design, tuning and assay of the final weapon pFW, obtained by**
405 **putting together the pathogenicity and antibiotic resistance (ABR) modules in**
406 **a single conjugative vector.** (a) Schematic representation of the specific killing of
407 *V. cholerae* O139 after pFW conjugation (left). Schematic display of the
408 corresponding AND-logic gate (right). (b) Conjugation from β3914 of either pN_{ctrl} or
409 pFW, of *V. cholerae* serogroup O139 (blue) and O1 (white) as recipient mixed
410 population. Transconjugants were selected on MH + Sp (plasmid marker). pFW
411 plasmid was obtained after change in RBS sequence of *ompU* promoter to increase
412 translation of toxin-intein fusion and substitution of the O4 operator sequence by O1
413 operator sequence (see online methods) to increase SetR binding affinity to the PL
414 promoter, and consequently increase repression. Only *V. cholerae* serogroup O1
415 that is devoid of SXT in its genome was detected after pFW conjugation,
416 demonstrating the specific killing of serogroup O139, which contain both
417 chromosomally encoded ToxR and SetR the chosen indicators of pathogenicity and

antibiotic resistance, respectively. Pictures are representative from three independent experiments.

Figure 4. Specific killing of pathogenic and ABR *V. cholerae* O139 in the zebrafish larvae and *Artemia salina* nauplii models. (a) Four-day-postfertilization zebrafish larvae were exposed to water containing 10^4 CFU/ml *V. cholerae* O139 or a mixed population containing 10^5 CFU/ml *V. cholerae* O139 + *V. cholerae* O1, and then infected (see methods) with 10^7 (O139) or 10^6 (mix *Vibrio*) CFU/ml of β3914 as donor strain of either pN_{ctrl} or pFW plasmids. Five larvae were fished and mashed to analyze its microbiota. Transconjugants were selected in MH media with Sp and X-gal. Transconjugants were only detected after conjugation with pN_{ctrl} plasmid for O139 and not after pFW conjugation as expected from the specific killing. Confirming pFW specifity, pFW transconjugants were detected for *V. cholerae* O1, which should not be killed by this plasmid. Data for O139 represent transconjugants obtained from 15 larvae fished in three independent experiments (n=3, mean ± s.d), and data from the mix of *Vibrio* represent transconjugants obtained from 10 larvae in two independent experiments (n=2, mean ± s.d). (b) *Artemia salina* stage nauplii were infected with 10^7 CFU/ml *V. cholerae* O139 or a mix of 10^7 CFU/ml *V. cholerae* O139 + *V. cholerae* O1 (see methods). Then exposed to 10^7 CFU/ml β3914 as donor strain of either pN_{ctrl} or pFW plasmids. Transconjugants were selected in MH media with Sp and X-gal. As in zebrafish, transconjugants were only detected after conjugation with pN_{ctrl} plasmid for O139 and not after pFW conjugation. As expected, *V. cholerae* O1 pFW transconjugants were also detected in this in vivo model. Data numbers were calculated from four independent experiments (n=4, mean ± s.d).

ONLINE METHODS

Strains and culture conditions.

Unless otherwise noted, bacterial cultures were grown at 37ºC with Luria-Bertani (LB) medium (Lennox) or Mueller-Hinton (MH) solid media supplemented when appropriate, with the following antibiotics: 50 µg/ml kanamycin (Kan), 50 µg/ml chloramphenicol (Cm), 100 µg/ml carbenicillin (Carb), 50 or 100 µg/ml spectinomycin (Sp) for *E. coli* and 100 µg/ml Sp for *Vibrio cholerae*. Selection of transconjugants was carried using 100 µg/ml Sp in all cases, except for *V. mimicus* and *V. vulnificus* where we used 50 µg/ml Sp. Bacterial strains used in this study are listed in Supplementary Table 1. Other molecules were added to the media with the following concentrations: 40 µg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Xgal), 0.3 mM Diaminopimelic acid (DAP), 1% glucose and 0,2% arabinose.

Plasmid construction.

Plasmids are listed in Supplementary Table 1 and primers in Supplementary Table 6. All plasmid sequences were verified through sequencing.

To generate the N and C plasmids for each toxin-intein fusion, the N- and C-terminal toxin regions were amplified with primers F-toxin-EcoRI/R-toxin-intein and F-toxin-intein/R-toxin-XbaI, respectively. N-and C-terminal intein regions were amplified with primers F-intein-toxin/R-intein-XbaI and F-intein-EcoRI/R-intein-toxin, respectively. As DNA templates for toxins we used chromosomal DNA from *V. cholerae* in all cases and *V. fischeri* for *ccdB*. Intein amplification was done with chromosomal DNA from the cyanobacteria *Nostoc punctiforme*. PCR products of N- and C-terminal regions were fused by Gibson assembly[35]. Each toxin-intein fusion was then digested with EcoRI/XbaI (Thermo Fisher) and then cloned in EcoRI/XbaI digested pBAD43[36] and pSU38[37] (or pSU18) plasmids, respectively (Supplementary Table 1). To generate the

mutated version of N-terminal plasmid (n*) whole plasmids were amplified using primers F-Int-tox-mut/R-int-tox-mut.

To assemble the pU-BAD plasmid (Supplementary Fig. 5) we first cloned the C-terminal CcdB-Npu fusion into a pBAD18 plasmid (EcoRI-XbaI). An *ompU* promoter was inserted upstream the N-terminal ccdB/Npu fusion in N plasmid by PCR. The *ompU* promoter region was amplified using F-PompU-1/R-PompU-dB (size=352 bp). This promoter was chosen based on previous work[21] that showed its high induction in the presence of ToxR. A region containing the pSC101 origin was amplified using R-BAD43-BAD18/F-4126 primers and the N-terminal CcdB-Npu plasmid as template. A second region containing the N-terminal fusion was amplified using 4217/R-BAD43-BAD18 primers and N-terminal plasmids also as templates. Other regions containing the C-terminal fusion and Kan resistance gene were amplified using R-BAD18-BAD43/F-BAD18-BAD43 primers and the C-terminal CcdB-Npu pBAD18 plasmid as template. PCR products were then fused by Gibson assembly[35] producing the pU-BAD plasmid.

To generate the pRS plasmid, (Supplementary Fig. 5) the *toxRS* operon from *V. cholerae* O1 was amplified using F-toxR-SacI/R-toxS-XbaI primers, digested with SacI and XbaI and ligated with SacI-XbaI digested plasmid pBAD30. The native RBS sequence of *toxR* was kept.

To assemble the toxin-intein N and C-terminal fusions as an operon (pToxInt plasmid), N- and C-fusions were amplified using F-CcdB-EcoRI/R-Int-N-Int-C and F-Int-C-Int-N/R-Int-XbaI primers and then ligated by Gibson assembly[35], digested with EcoRI/XbaI and cloned into a pBAD43-EcoRI/XbaI digested plasmid. The fusion contains the following sequence: 5' TGATAAGGAGGTAACATATG 3' between the N and C genes. This sequence contains the RBS sequence necessary for translation of the C-terminal fusion. The pTox plasmid was created by amplification of the *ccdB* toxin gene from *V. fischeri* DNA with F-CcdB-EcoRI/R-CcdB-XbaI primers, EcoRI/XbaI digestion and ligation into a pBAD43-EcoRI/XbaI digested plasmid. *E. coli* XL2blue strain that

contains F' plasmid integrated in the chromosome (containing the *ccdB/ccdA* TA system and conferring resistance to CcdB), was used to transform with this ligation in order to obtain positive clones.

To assemble the pPW genetic weapon, the *ompU* promoter was amplified as previously described and ligated by Gibson assembly[35] with the product of pToxInt plasmid PCR using F-dB-PompU/R-BAD-PU1 primers.

The pPLA plasmid was constructed first by amplifying by PCR the PL promoter[38] using DNA from *V. cholerae* O139 and F-PL-plasmid/R-PL-ccdA as primers. Then, the *ccdA* antitoxin gene was amplified using the F-ccdA-PL/R-ccdA-plasmid primers and *V. fischeri* DNA. Finally, the pTox-Int plasmid was also amplified using F-plasmid-dA/R-plasmid-PL primers. Ligation by Gibson assembly[35] of the three PCR products resulted in the pPLA plasmid.

Mobilizable genetic weapons were created by amplifying the origin of transfer *oriT* RP4 using F-pSW23-BAD/R-oriT-BAD43 primers and the plasmid pSW23T[39] as template. Then, the *oriT* PCR product was ligated through Gibson assembly[35] with the amplified plasmid using F-BAD-pSW/R-BAD43-oriT primers and the weapon or control plasmids as template.

To assemble the Final Weapon we the plasmid pFW (Figure 3) as follow. The *ompU* promoter-1 was ligated into the pABRW plasmid as previously described for the pU-BAD construction. In order to fine-tune the RBS of *ompU* in this plasmid as well as the PL promoters,, PCRs were performed using F-ccdB-SD-OK/R-PU-SD-OK and F-PL-SD-T/R-PL-SD-T primer pairs, respectively. Finally, to generate the pFW plasmid, an operator O1 sequence (see [38]) was added into the PL promoter by PCR amplification of the pFW2 plasmid using F-PL-O1/R-PL-O1 primers.

To generate the pPW-R6K, pFW-R6K and pNctrl-R6K plasmids we first amplified the R6K replication origin using F-R6K-weapon/R-R6K-weapon primers and the pMP7[40] plasmid as template. Then, the pPW, pFW and pNctrl plasmids were amplified using F-

weapon-R6K/R-weapon-R6K primers. Finally, PCR fragments were ligated by Gibson assembly[35].

Δ*toxRS* strain construction

DNA regions 500 bp upstream and downstream of the *toxRS* operon were amplified using F-toxRup-p7/R-toxRups and F-toxSdow/R-toxSdow-p7, respectively. The amplified fragments were ligated by Gibson assembly[35] and then cloned into an R6K γ-*ori*-based suicide vector, pSW7848[40] that encodes the *ccdB* toxin gene under the control of an arabinose-inducible promoter, P_{BAD}. For conjugal transfer of plasmids into *V. cholerae* strains, *E. coli* β3914 was used as the donor. Clones where integration of the entire plasmid in the chromosome by single crossover occurred were selected. Elimination of the plasmid backbone resulting from a second recombination step was selected as described ref 39.

Transformation assays

DH5α chimiocompetent cells (Invitrogen) were transformed with 150 ng of pTox, pToxInt or pN plasmids (Supplementary Fig. 1a). Transformants were then tested in Sp containing media with glucose or arabinose to analyze toxin integrity. 10 to 12% of pTox-transformed clones from were able to grow in the presence of arabinose. Four independent clones were analyzed by sequencing and they all carried an insertion sequence in the *ccdB* toxin gene. These clones were responsible for pTox transformation rate decrease in comparison with the pToxInt and pN plasmids.

DH5α cells (Invitrogen) were co-transformed with two plasmids simultaneously. Both plasmids were then simultaneously selected (Supplementary Fig.4).

Transformation of the donor strain β3914 was performed in the presence of DAP.

Growth tests

Eighteen independent clones from DH5α transformation were inoculated in p96 microplates containing LB media with Sp and glucose. The TECAN Infinite 200 microplate reader (TECAN, Männedorf, Germany) was used to determine growth curves, with absorbance (620nm) taken at 6-minute intervals for a period of 12 h. The obtained OD values were plotted as seen on Supplementary Fig. 1b.

In Supplementary Fig. 2 for analysis of bactericide effect of CcdB toxin: *V. cholerae* O139 was co-transformed with antitoxin-*ccdA (*pBAD24-*ccdA)* and pPW plasmids in the presence of arabinose allowing the antitoxin to be expressed. pPW plasmid contains the toxin-intein under the control of *ompU* promoter, which is always active in *V. cholerae*. Bacteria culture supplemented with antibiotics for maintaining both plasmids and arabinose, were diluted at OD=0.5 (time 0h). Then bacteria were washed three times with MH media with antibiotics and glucose, in order to switch off antitoxin expression, and incubated for 4h at 37ºC. Total bacteria were calculated by the CFU/ml at time 0h and 4h present in MH media with antibiotics and with glucose (1%) or arabinose (0,2%). Data numbers were calculated from four independent experiments (n=4).

Conjugation assays.

Overnight cultures of donor and recipient strains were diluted 1:100 in culture media with antibiotic and grown at 37ºC for 2-3 hours. Then, cultures were diluted to an OD_{600} = 0.5. The different conjugation experiments were performed by a filter mating procedure described previously[41] with a donor/recipient ratio of 1::1. When the recipients were composed of a mixed population the donor/mixed-recipient ratio was 1::0.5-0.5. Before mixing the different bacteria, cultures were washed three times with fresh media to remove antibiotics. In Supplementary Table 2 bacteria were mixed in different proportions (2:1 and 3:1) to test whether this would impact conjugation efficiency. Conjugation was performed during 4h at 37ºC on filter in MH plates

supplemented with DAP (and containing NaCl until 332mM final concentration in the case of *V. vulnificus*).

In vivo **conjugation in zebrafish larvae and** ***Artemia salina***

All animal experiments described in the present study were conducted at the Institut Pasteur according to European Union guidelines for handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and were approved by the Institut Pasteur Animal Care and Use Committee and the Direction Sanitaire et Veterinaire de Paris under permit #A-75-1061. Conjugative killing was assessed as follow. Four-day-postfertilization zebrafish larvae were exposed to water containing 10^4 CFU/ml of *V. cholerae* O139 for 2 hours at 27ºC (Figure 4a) or a 1::1 mixed population containing 10^5 CFU/ml *V. cholerae* O139 + *V. cholerae* O1 (Figure 5a, mix *Vibrio*) for 2 hours at 27ºC. Then, larvae were washed in sterile water three times and then placed into a well containing 10^7 or 10^6 CFU/ml (Figure 4a, *V. cholerae* O139 and mix *Vibrio*, respectively) of the *E. coli* β3914-Δ*dap* donor strain containing either the pN_{ctrl} or pFW plasmid for 24 hours at 27ºC. In Supplementary Fig. 11b and 11c, infection dose for *Vibrio* was the same than for Fig. 4a. Larvae were transferred to bacteria-free wells, washed in sterile water three times and then placed into a well containing Tricaine (Sigma-Aldrich #E10521) at 200 mg/ml to euthanize them. Finally, they were transferred to a tube containing calibrated glass beads (acid washed, 425 um to 600 um, Sigma-Aldrich #G8722) and 500 µl of water. Five larvae were mashed using FastPrep® Cell Disrupter (BIO101/FP120 QBioGene) for 45 seconds at maximum speed (6,5 m/sec) to analyze their microbiota (Supplementary Fig. 11) in MH Media + X-gal or TCBS media for selection of *V. cholerae*. Blue bacteria corresponding to *V. cholerae* O139 were detected in MH media. Transconjugants selection was done into MH Media + X-gal and Sp and then, replication of these MH plates were done on TCBS media to specific identify *V. cholerae*. Strain identity was confirmed through

yellow color development in TCBS *Vibrio* specific media. The amoeba *Tetrahymena thermophila* (*T. thermophila*) was added to feed larvae during the experiment.

Groups of 225±15 larvaes of *Artemia salina* stage nauplii suspended in 1ml volume of seawater were washed using sterile cell strainer Nylon filters 100 µm pore size (Falcon) and three times with the same volume (3x1ml) of sterile PBS (D8537, Sigma). Nauplii were suspended in 1ml PBS and then infected with 10^7 *V. cholerae* O1 or a mix of 10^7 *V. cholerae* O1 and O139 for 2 hours in agitation at 27ºC. Then nauplii were washed as previously described and exposed to 10^7 of β3914 -Δ*dap* bacteria with pN_{ctrl} or pFW plasmid for 4 hours at 27ºC. These experiments were repeated four times independently. The microbiota from 1ml containing 225±15 nauplii were analyzed as previously described for zebrafish. In the case of *Artemia*, we have used M63B1 minimal media where *Artemia* feel asleep and then put them on ice, previous the use of fast-prep (FastPrep® Cell Disrupter (BIO101/FP120 QBioGene) for 45 seconds at maximum speed (6,5 m/sec)). Transconjugants were selected from 225±15 nauplii after pN_{ctrl} or pFW conjugation treatment into MH media with Sp and X-gal (Supplementary Fig. 12a,b). For the identification of *V. cholerae* in the mix of both serogroups (Fig. 4b and Supplementary Fig. 12b), replication of these MH plates were done into TCBS media to specificaly identify *V. cholerae*. Strain identity was confirmed through yellow color development in TCBS *Vibrio* specific media.

Co-localization of *E. coli* and *V. cholerae* in the zebrafish larvae and *A. salina* by Microscopy.

Co-localization of *E. coli* and *V. cholerae* in the zebrafish larvae was assessed as follow. Four-day-postfertilization zebrafish larvae were exposed to water containing 10^6 CFU/ml *V. cholerae O1*-GFP for 2 hours at 27ºC. Then washed in sterile water three times and then placed into a well containing 10^7 CFU/ml of *E. coli*-RFP for 24 hours at 27ºC. Larvae were removed from the well and then placed into a well containing

Tricaine for euthanize them. Infected and non-infected larvae were visualized by fluorescence microscopy (EVOS FL microscope-Life technologies) using appropriate wavelength conditions enabling or not the visualization of GFP and RFP. Fluorescence was only detected in infected larvae and more precisely into the gut where both bacteria are co-localized.

In the case of *A. salina* stage nauplii the microscopy experiment was done using 10^7 *V. cholerae*-GFP for 2 hours in agitation at 27ºC. Then nauplii were washed as previously described and exposed to 10^7 of *E. coli*-RFP strain for 2 hours. Microscopy conditions were performed as for zebrafish experiment.

Statistics

In Supplementary Fig. 9, one-way ANOVA with Dunnett's Multiple Comparison Test was performed. PNcontrol-R6K vs pPW-R6K, Mean Diff. = 2.383e+008, q = 4.183, **P<0.05, 95% CI of diff = (8.937e+007 to 3.871e+008). PNcontrol-R6K vs pFW-R6K, Mean Diff. = 2.308e+008, q = 4.227, **P<0.05, 95% CI of diff = (9.187e+007 to 3.896e+008).

In Supplementary Fig. 13, one-sided t-test Mann Withney was performed. *E. coli* SXT vs *E. coli* MG1655. P value = 0.0143. *P < 0.05.

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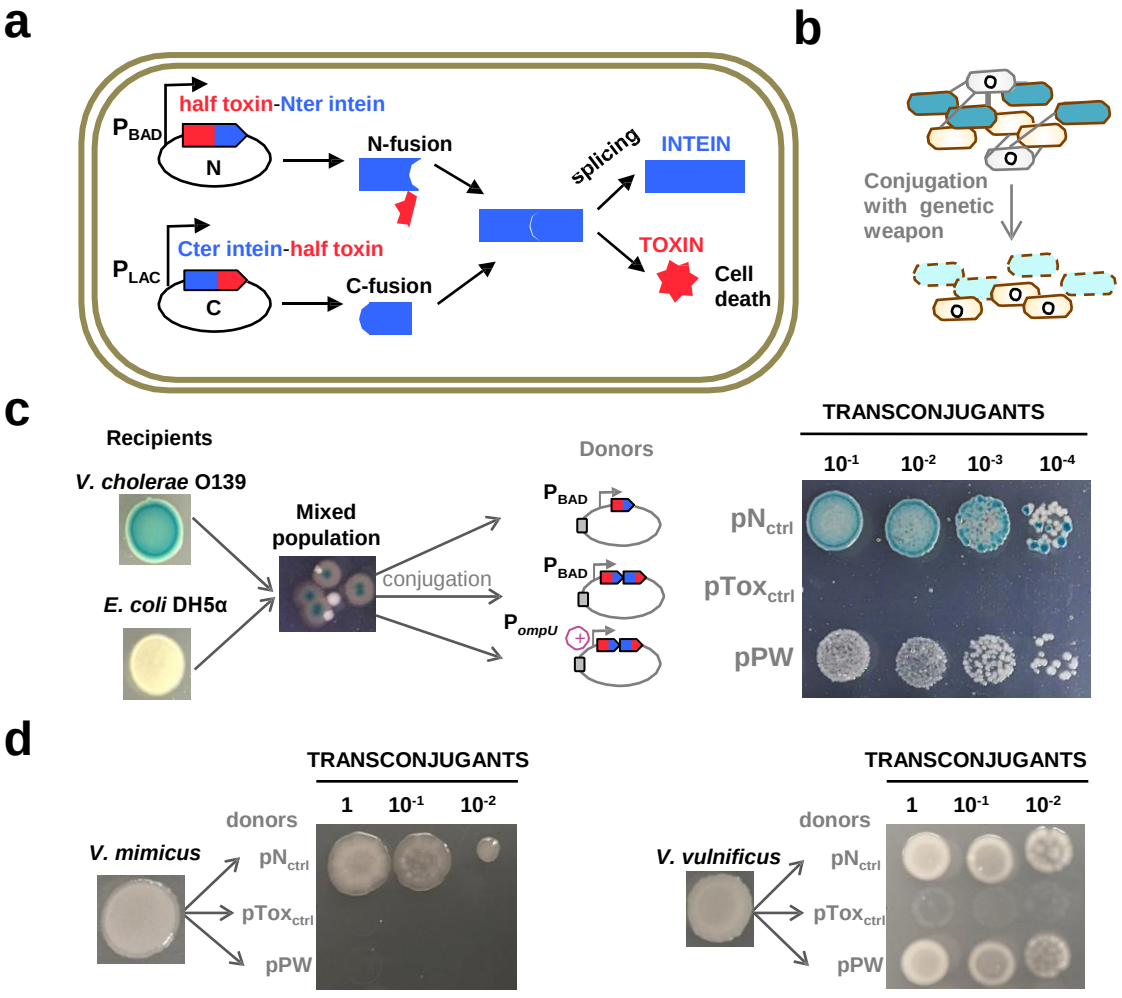

a
half toxin-Nter intein
P BAD
N
N-fusion
splicing
INTEIN
Cter intein-half toxin
P LAC
C
C-fusion
TOXIN
Cell death
b
Conjugation with genetic weapon
c
Recipients
V. cholerae O139
E. coli DH5α
Mixed population
conjugation
Donors
P BAD
P BAD
P ompU
TRANSCONJUGANTS
10-1 10-2 10-3 10-4
pNctrl
pToxctrl
pPW
d
TRANSCONJUGANTS
1 10-1 10-2
donors
V. mimicus
pNctrl
pToxctrl
pPW
TRANSCONJUGANTS
1 10-1 10-2
donors
V. vulnificus
pNctrl
pToxctrl
pPW

Figure 1

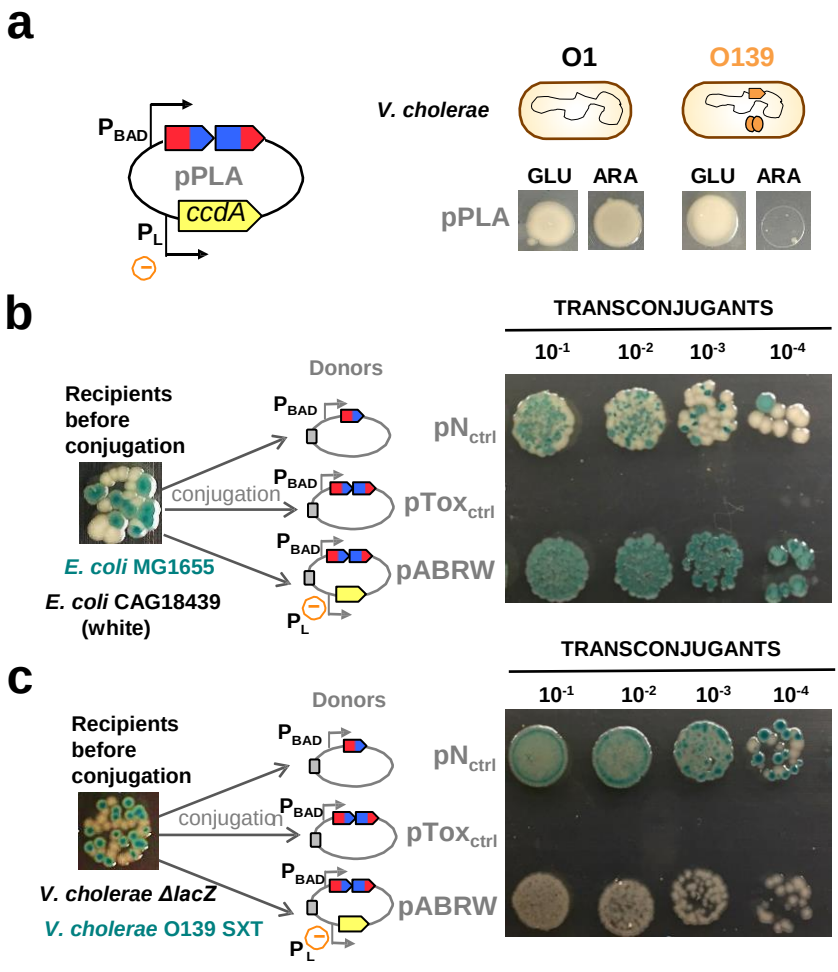

a
P BAD
pPLA
ccdA
P L
O1
O139
V. cholerae
GLU
ARA
GLU
ARA
pPLA
b
TRANSCONJUGANTS
10-1
10-2
10-3
10-4
Donors
Recipients
before
conjugation
conjugation
pNctrl
pToxctrl
pABRW
E. coli MG1655
E. coli CAG18439
(white)
c
TRANSCONJUGANTS
10-1
10-2
10-3
10-4
Donors
Recipients
before
conjugation
conjugation
pNctrl
pToxctrl
pABRW
V. cholerae ΔlacZ
V. cholerae O139 SXT

Figure 2

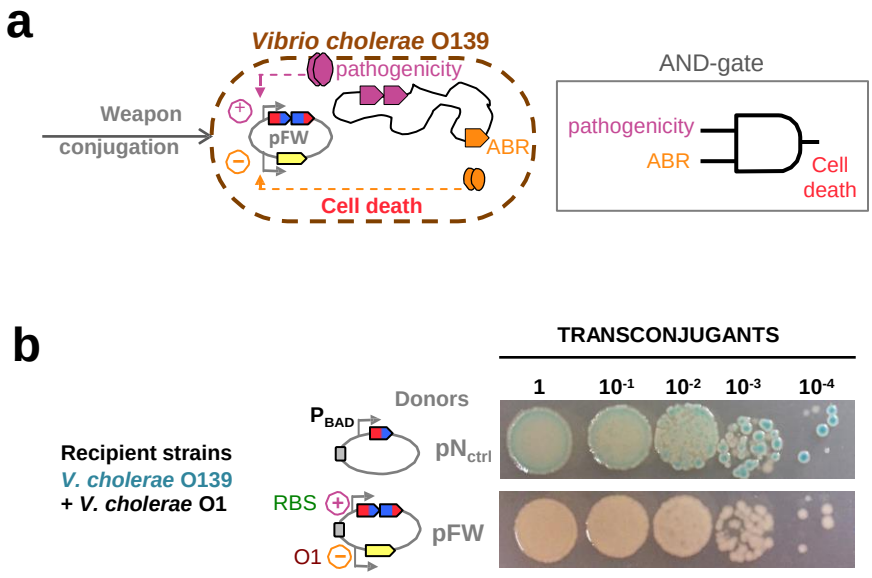

a
Vibrio cholerae O139
pathogenicity
Weapon
conjugation
pFW
ABR
Cell death
AND-gate
pathogenicity
ABR
Cell
death
b
TRANSCONJUGANTS
1
10-1
10-2
10-3
10-4
Donors
PBAD
pNctrl
RBS
pFW
O1
Recipient strains
V. cholerae O139
+ V. cholerae O1

Figure 3

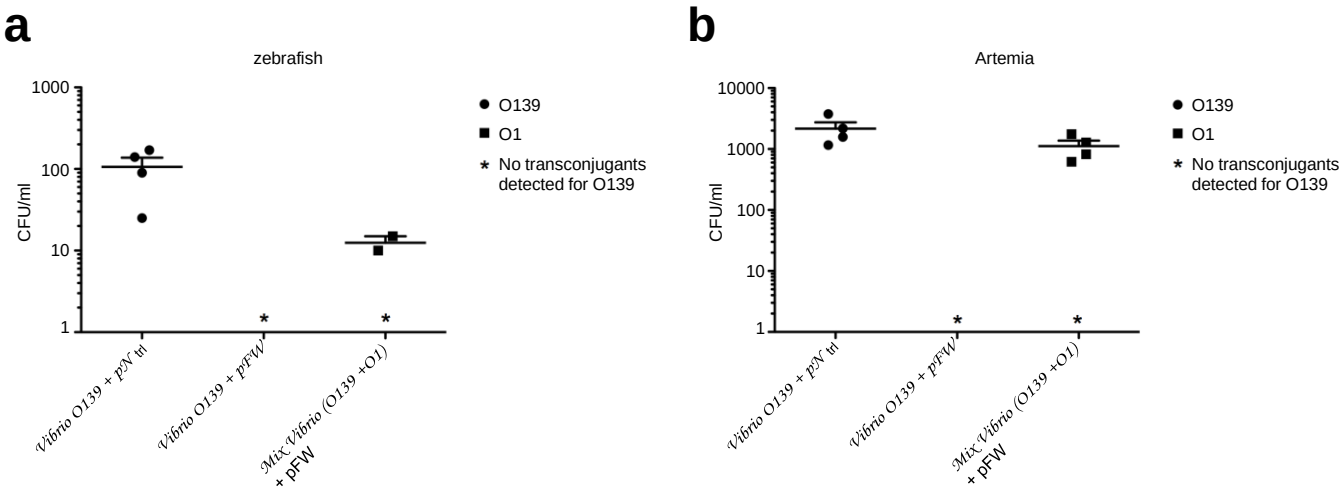

a
zebrafish
1000
100
10
1
CFU/ml
O139
O1
No transconjugants detected for O139
Vibrio O139 + pVtl
Vibrio O139 + pFW
Mix Vibrio (O139 +O1) + pFW
b
Artemia
10000
1000
100
10
1
CFU/ml
O139
O1
No transconjugants detected for O139
Vibrio O139 + pVtl
Vibrio O139 + pFW
Mix Vibrio (O139 +O1) + pFW

Figure 4