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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* present in mixed populations. We find that 100% of antibiotic-resistant *V. cholerae* receiving the plasmid are killed. Escape mutants were extremely rare (10^{-6}–10^{-8}). We show that conjugation and specific killing of targeted bacteria occurs in the microbiota of zebrafish and crustacean larvae, which are natural hosts for *Vibrio* spp. Toxins split with inteins could form the basis of precision antimicrobials to target pathogens that are antibiotic resistant.

With the advent of the antibiotic era, infectious diseases were thought to be under control, but a worldwide emergence of antibiotic resistant (ABR) bacteria has occurred, due to the widespread unchecked use of antibiotics. Further, it is now estimated that ABR bacteria could be the main cause of death by 2050 (ref. [1]) unless new classes of antimicrobials are developed.

Broad spectrum antimicrobials indiscriminately kill bacteria that can result in microbiota dysbiosis and concomitant health sequelae. Moreover, antibiotics that have nonspecific 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 ABR *Vibrio cholerae*. To mediate bacterial killing we chose the toxin component of type II bacterial toxin-antitoxin 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 reduced the potential for development of resistance. Each antitoxin is highly specific for the cognate toxin and non-specific toxin-antitoxin interactions were counterselected[10]. Our antimicrobial design relied on the regulation of type II toxin-antitoxin transcription by highly specific transcription factors. This meant that activation of the toxin and concomitant killing of individual members of mixed bacterial populations was feasible if a targeted bacterial species expressed the type II toxin-regulating transcription factor. We validated our approach by showing that we could selectively kill ABR *V. cholerae* present in mixed populations.

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

Our previous experience with type II toxins[14,15] taught us that basal expression of a full-length toxin gene from P_{BAD} is sufficient to kill the *E. coli* host. To avoid this, we designed a genetic module containing a toxin split by an intein, and in our module the split toxin–intein can be activated only by ToxR. Inteins are protein sequences embedded into a host protein (extein) from which they are autocatalytically excised in a process called protein splicing. During protein splicing, the intein ligates the extein extremities and allows the reconstitution of the mature protein. In nature, a few examples of split inteins also exist allowing the assembly of a single protein from two genes[16]. We split the type II toxin gene *ccdB* (Plasmid pToxInt, Supplementary Fig. 1) into two parts, each of which is associated with half of a split intein. Split inteins have been used in several biotechnological tools[17] and enable control of toxic protein functions in vivo[18]. We used the split-intein DnaE, which is present in the *dnaE* gene of *Nostoc punctiforme*. DnaE is well characterized and has a high rate of trans-splicing[19]. Using inteins enables strict control of toxin production and avoids toxicity due to basal expression[14,15] (Supplementary Fig. 1).

First, we cloned full-length gyrase inhibiting toxin CcdB from *Vibrio fischeri*[15] into a plasmid (pTOX, Supplementary Table 1) and transformed the toxin construct into a *E. coli* XL2 blue

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(Supplementary Table 1) that constitutively expresses a genomic copy of the cognate antitoxin (data not shown). We showed that *ccdB* was bactericidal (Supplementary Fig. 2) and that the intein-mediated splitting strategy led to more stable retention of the toxin-harboring plasmid under repression conditions compared with a construct harboring a whole *ccdB* toxin gene (Supplementary Fig. 1). We also evaluated whether three other type II toxins belonging to different toxin families (ParE2, HigB2 and RelE4, ref. [14]) could tolerate a splitting and stay functional. We selected intein insertion points by inspection of 3D structure predictions for toxins made in Phyre2 (ref. [20]), a tool for modeling protein structure (Supplementary Fig. 3a). Each toxin was divided into N and C terminal portions (Supplementary Fig. 3b), which were fused in-frame to the N or C parts of the split-intein *dnaE* gene (102 and 36 amino acids long), respectively. N and C terminal toxin–intein fusions were cloned in separate, compatible plasmids (N or C plasmids, respectively, Supplementary Table 1) and were under the control of different promoters (Fig. 1a). We validated reconstitution of the active toxin by intein protein splicing in *E. coli* (Supplementary Fig. 4). For all five tested split toxins, we found that under inducing conditions bacteria containing N and C plasmids died, whereas bacteria with either the N or the C plasmid survived. N and C toxin–intein complex toxicity was tested using mutations known to prevent splicing. When splicing did not occur, reconstitution of the toxin did not take place and bacteria survived (Supplementary Fig. 4).

Next, we chose the gyrase poison CcdB, which is probably the most extensively characterized type II toxin, to design a toxin–intein antimicrobial specific for pathogenic *V. cholerae*. In *V. cholerae* one of the ToxRS-regulated genes encodes a membrane porin, OmpU[21]. We cloned the N fusion of CcdB-intein downstream of the *ompU* promoter (regulated by ToxRS) and the C fusion under P_{BAD} in the same plasmid (pU-BAD, Supplementary Fig. 5a). The functionality of pU-BAD was tested in an *E. coli* DH5α strain expressing the *V. cholerae toxRS* operon from a second plasmid (pRS, Supplementary Fig. 5a). On arabinose-mediated induction of *toxRS* expression, only bacteria containing both pU-BAD and pRS plasmids died (Supplementary Fig. 5b). We replicated cell killing in 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* (*ΔtoxRS*) 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*.

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 and Supplementary Table 1). Conjugation is carried out from donor strain *E. coli* β3914, an MG1655 *ΔdapA* that contains the RP4 conjugative machinery integrated into its chromosome. pPW was introduced by conjugation into *V. cholerae* strains O1, O139 and an O1-*ΔtoxRS* mutant (Supplementary Fig. 6b), but only the *ΔtoxRS* 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 the $pTox_{ctrl}$ plasmid, which carries the P_{BAD}-regulated toxin–intein operon) into this mixture. After conjugation of pPW from *E. coli* β3914 and selection for transconjugants, pPW killed *V. cholerae* O139 (blue bacteria) and we were only able to detect *E. coli* DH5α transconjugants (white) on media containing X-Gal. Similarly, after plasmid conjugation into *V. cholerae* O1 and *E. coli* strains (MG1655), we only obtained *E. coli* transconjugants (Supplementary Fig. 7a).

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

Next, we evaluated whether a split-intein toxin could kill ABR bacteria present in a community. The SXT integrative and conjugative element family in *V. cholerae* includes various antibiotic resistance genes[13]. The SXT chassis encodes several transcription factors that regulate SXT transmission including the SetR repressor[13]. We designed a module to detect SXT carriage and kill SXT-harboring bacteria by implementing an additional component into our antimicrobial: the *ccdA* gene, which encodes the antitoxin partner of CcdB. *ccdA* was cloned downstream of the SXT PL promoter, which is controlled by the SetR repressor, in a plasmid also containing the *ccdB*-intein operon regulated by the P_{BAD} promoter (pPLA plasmid, Supplementary Fig. 8a and Supplementary Table 1). We tested whether pPLA could kill ABR *E. coli* SXT (Supplementary Fig. 8b) and *V. cholerae* O139 (Fig. 2a). Both bacteria contain an SXT element integrated at *prfC*. Only SXT carrying bacteria from both species were killed. All bacteria lacking SXT, including *V. cholerae* O1 and *E. coli* DH5α, survived (Fig. 3a and Supplementary Fig. 8b). To develop a conjugative antimicrobial to kill ABR bacteria we added an *oriT* to pPLA to produce pABRW (Supplementary Table 1 and Fig. 2). pABRW was tested by conjugation into a mixed population of *E. coli* MG1655 (blue) and *E. coli* SXT (white). Selection for pABRW yielded only *E. coli* MG1655 transconjugants, demonstrating that pABRW specifically kills bacteria containing SXT (Fig. 2b). The same result was obtained after conjugation of pABRW into *V. cholerae* O139 mixed with *V. cholerae* O1-*ΔlacZ* (Fig. 2c), confirming that pABRW plasmid specifically kills ABR bacteria in a heterogeneous population.

We next combined the pPW and pABRW modules in a single plasmid. We replaced the operator sequence O4 of PL with O1 (see Methods) to increase SetR repression to yield pFW (Supplementary Table 1 and Fig. 3), which efficiently kills *V. cholerae* O139 (Fig. 3b). To test whether non-replicative-conjugative plasmids (which would not spread toxin–intein fusions and/or ABR 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, amounts of colony-forming units (c.f.u.) per 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 limits the risk of unnecessary propagation of the killing plasmid.

We moved on to 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

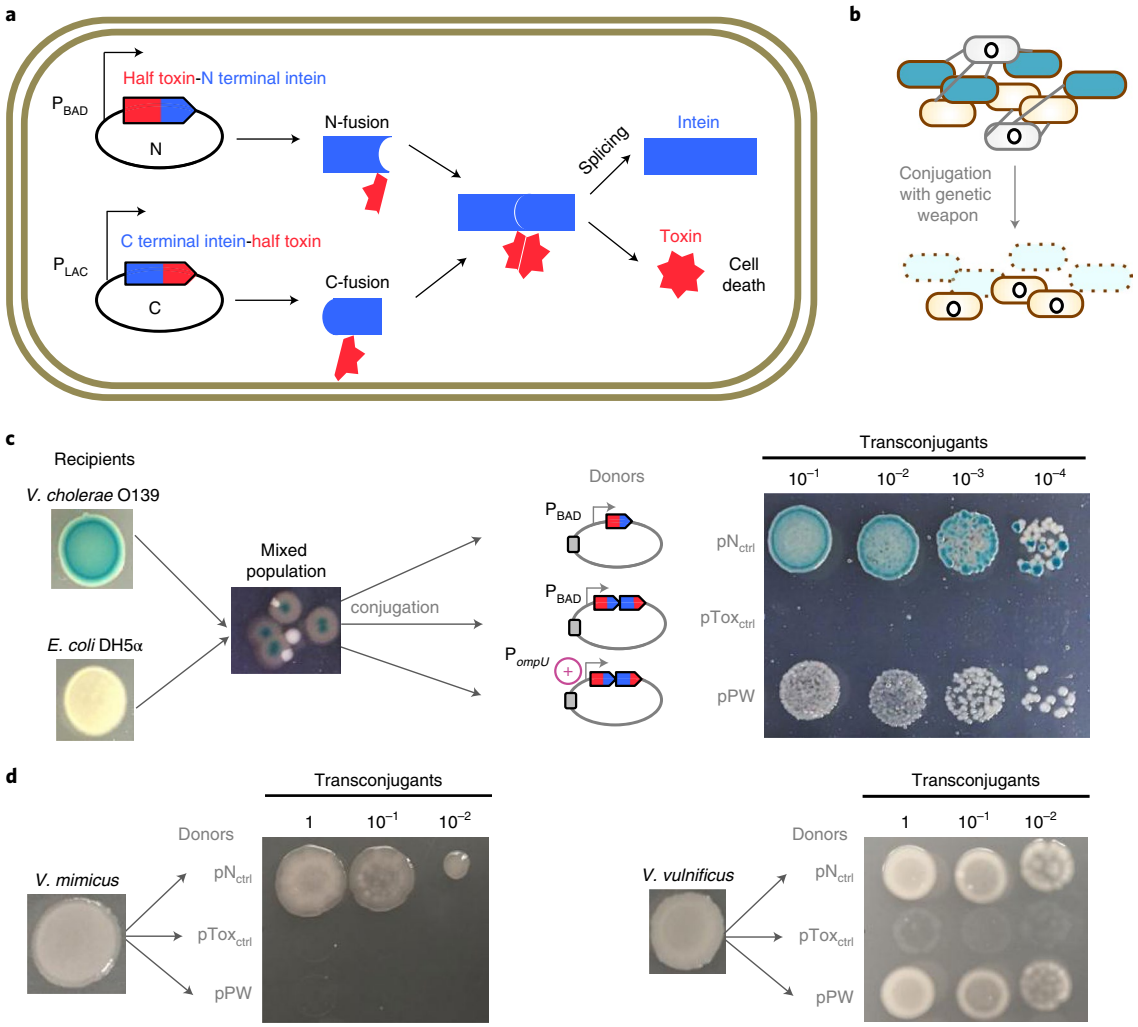

Fig. 1 | Specific killing of pathogenic *V. cholerae* in mixed population of bacteria mediated by toxin–intein strategy. a, Schematic representation of the active toxin production from plasmids encoding split toxin (red) combined with split intein (blue) inside a bacterium. The first half of the toxin 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 controlled by P_{LAC}. Expression of these fusions is activated by the addition of arabinose and isopropyl-b-D-thiogalactoside, respectively. Recognition of the protein fusions takes place by the intein module that carries out the splicing process, which leads to toxin reconstitution provoking cell death. **b**, Mode of action of the genetic weapon spreading through conjugation in a 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 the donor strain containing pN_{ctrl}, $pTox_{ctrl}$ or pPW plasmids. The *ompU* promoter activated specifically by ToxRS from *V. cholerae* is represented by a pink circle with a '+' symbol. Transconjugants were selected in Mueller–Hinton media + spectinomycin (Sp), X-gal for color development and arabinose for induction of P_{BAD}. **d**, Conjugation of pN_{ctrl}, $pTox_{ctrl}$ and pPW plasmids using β3914 as donor strain in *Vibrio mimicus* and *Vibrio vulnificus*. Transconjugants were selected in Mueller–Hinton media + Sp and arabinose for induction of P_{BAD}. Pictures are representative of three independent experiments.

for the diaminopimelic acid (DAP) for delivering conjugative plasmid pNctrl, in the 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 when receiving the pFW, *V. cholerae* was killed. These preliminary data indicate 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 four-day post-fertilization zebrafish larvae detected fewer than 30 different bacterial species, mostly aerobic, including several *Aeromonads*, *Pseudomonads* and *Stenotrophimonads* (J.B.-B. and J.-M.G., 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*-red fluorescent protein (RFP). Fluorescence microscopy revealed co-localization of both *V. cholerae* O1-green fluorescent protein (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 and 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 killed *V. cholerae* O139 in zebrafish larvae (Fig. 4a and Supplementary Fig. 11b). We assessed dysbiosis using observation after plating on different media and did not 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

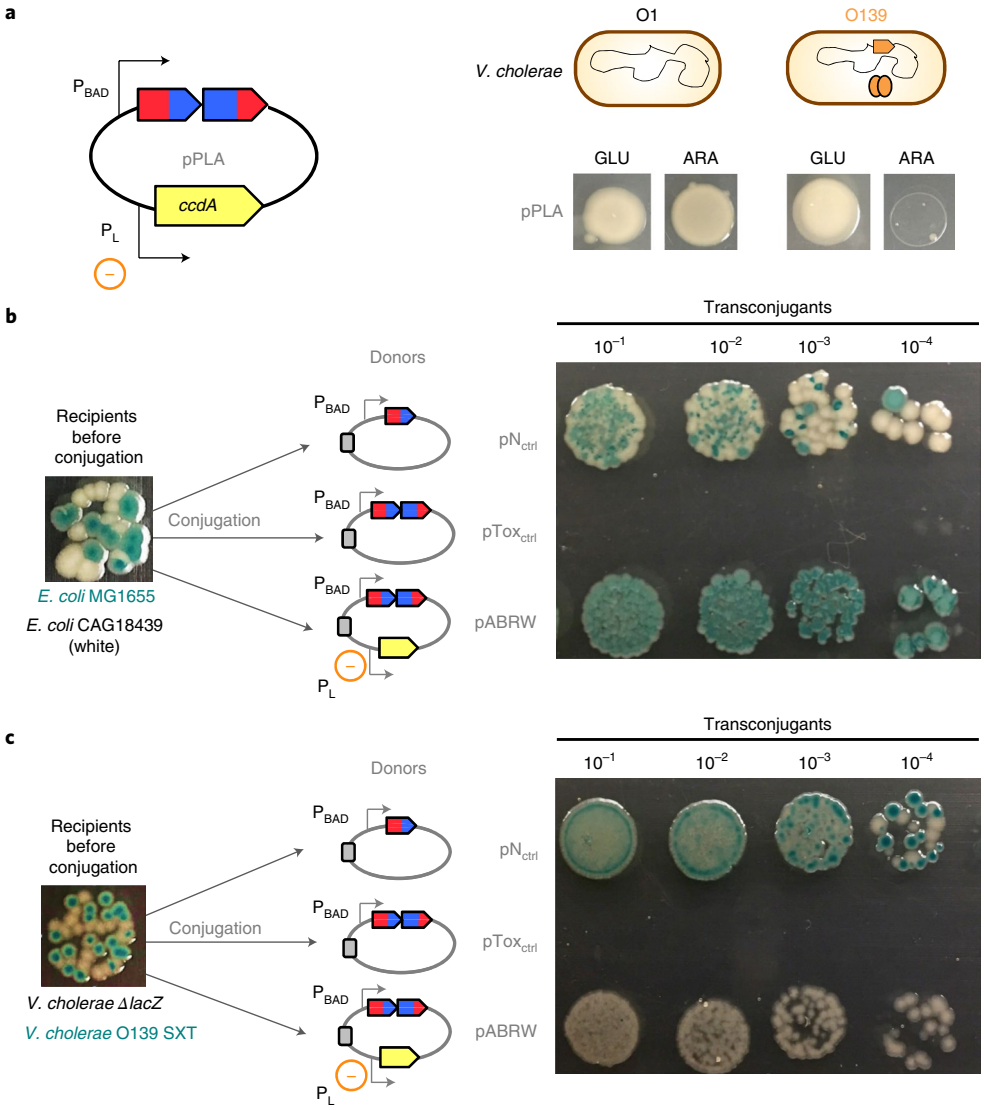

Fig. 2 | Specific killing of ABR bacteria (containing SXT). a, pPLA plasmid that contains CcdB-intein fusion operon under P_{BAD} control expression and *ccdA* antitoxin under a PL promoter (symbolized by an orange circle with a '−' symbol), which is repressed by SetR. Growth test of *V. cholerae* O1 or O139 containing pPLA plasmid in Mueller–Hinton media + Sp and supplemented with glucose (GLU) or arabinose (ARA). **b**, Mixed population of *E. coli* MG1655 (blue) and *E. coli* SXT (CAG 18439, white) as recipients for conjugation using β3914 as donor strain containing pN_{ctrl}, $pTox_{ctrl}$ or pABRW plasmids. Transconjugants were selected on Mueller–Hinton media + Sp, X-gal for species identification and arabinose for induction of P_{BAD}. **c**, Mixed population of *V. cholerae* O139-SXT (blue) and *V. cholerae* O1 Δ*lacZ* (white) as recipients for conjugation using β3914 as donor strain containing pN_{ctrl}, $pTox_{ctrl}$ or pABRW plasmids, as described in **b**. Pictures are representative of three independent experiments.

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 *A. salina* model that is used for fish feeding and commonly found to carry various *Vibrio* species[26] (Fig. 4b and Supplementary Figs. 10b and 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 transcription factors are widespread in pathogenic and ABR bacterial pathogens[30,31]. The Achilles' heel of precision antimicrobials is delivery into complex communities. Antimicrobials delivered by conjugation, for example RNA-guided nucleases[5], have reduced targeted bacterial populations by 2–3-log even with a ratio of donor/recipient bacteria of 340:1 (ref. [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

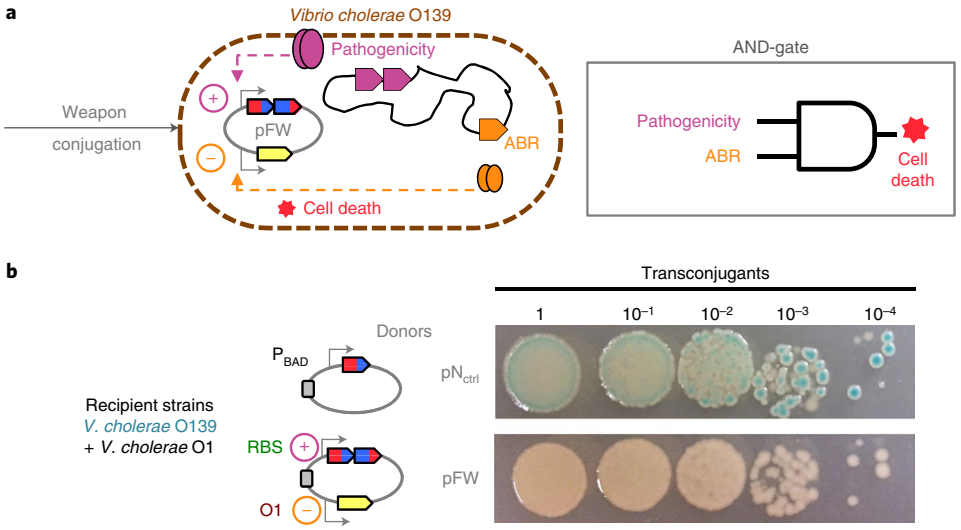

Fig. 3 | Design, tuning and assay of the Final Weapon pFW, obtained by putting together the pathogenicity and ABR modules in a single conjugative vector. a, Schematic representation of the specific killing of *V. cholerae* O139 after pFW conjugation (left). Schematic display of the corresponding AND-logic gate (right). **b**, Conjugation from β3914 of either pN_{ctrl} or pFW, of *V. cholerae* serogroup O139 (blue) and O1 (white) as a recipient mixed population. Transconjugants were selected on Mueller–Hinton + Sp (plasmid marker). The pFW plasmid was obtained after a change in a ribosomal binding site (RBS) sequence of *ompU* promoter to increase translation of toxin–intein fusion and substitution of the O4 operator sequence by O1 operator sequence (see Methods) to increase SetR binding affinity to the PL promoter and consequently increase repression. Only the *V. cholerae* serogroup O1, which is devoid of SXT in its genome, was detected after pFW conjugation, demonstrating the specific killing of serogroup O139, which contains both chromosomally encoded ToxR and SetR, the chosen indicators of pathogenicity and antibiotic resistance, respectively. Pictures are representative of three independent experiments.

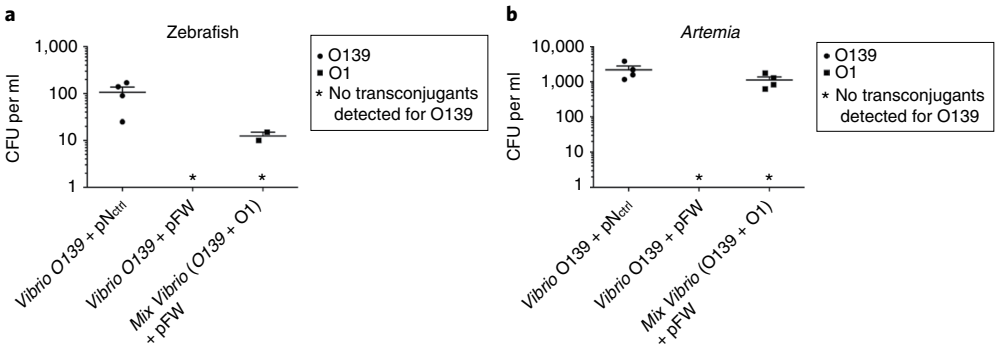

Fig. 4 | Specific killing of pathogenic and ABR *V. cholerae* O139 in zebrafish larvae and *Artemia salina* nauplii models. a, Four-day-post-fertilization zebrafish larvae were exposed to water containing 10^4 c.f.u. per ml *V. cholerae* O139, or a mixed population containing 10^5 c.f.u. per ml *V. cholerae* O139 + *V. cholerae* O1, and then infected (see Methods) with 10^7 (O139) or 10^6 (mix *Vibrio*) c.f.u. per ml of β3914 as donor strain of either pN_{ctrl} or pFW plasmids. Five larvae were fished and mashed to analyze their microbiota. Transconjugants were selected in Mueller–Hinton 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 specificity, 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 ten larvae in two independent experiments ($n = 2$, mean ± s.d). **b**, *A. salina* stage nauplii were infected with 10^7 c.f.u. per ml *V. cholerae* O139 or a mix of 10^7 c.f.u. per ml *V. cholerae* O139 + *V. cholerae* O1 (see Methods). They were then exposed to 10^7 c.f.u. per ml β3914 as donor strain of either pN_{ctrl} or pFW plasmids. Transconjugants were selected in Mueller–Hinton 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 were calculated from four independent experiments ($n = 4$, mean ± s.d).

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 toxin-antitoxin 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.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41587-019-0105-3.

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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 great participation from J.B.-B., J.-M.G. and A.R.-P.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Strains and culture conditions. Unless otherwise noted, bacterial cultures were grown at 37 °C with Luria–Bertani medium (Lennox) or Mueller–Hinton solid media supplemented when appropriate, with the following antibiotics: 50 μg ml^{-1} kanamycin (Kan), 50 μg ml^{-1} chloramphenicol (Cm), 100 μg ml^{-1} carbenicillin (Carb), 50 or 100 μg ml^{-1} spectinomycin (Sp) for *E. coli* and 100 μg ml^{-1} Sp for *V. cholerae*. Selection of transconjugants was carried out using 100 μg ml^{-1} Sp in all cases, except for *V. mimicus* and *V. vulnificus* where we used 50 μg ml^{-1} 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^{-1} 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Ggal), 0.3 mM 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 the N plasmid by PCR. The *ompU* promoter region was amplified using F-PompU-1/R-PompU-dB (size, 352 base pairs). This promoter was chosen on the basis of 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 a 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 a 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* toxin-antitoxin system and conferring resistance to CcdB), was used to transform with this ligation 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 a 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 a template.

To assemble the Final Weapon, we the plasmid pFW (Fig. 3) as follow. The *ompU* promoter-1 was ligated into the pABRW plasmid as previously described for the pU-BAD construction. 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 ref. [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 (ref. [40]) plasmid as a 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 (ref. [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 in 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. Then, 10–12% 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 was used to determine growth curves, with absorbance (620 nm) taken at 6-min intervals for a period of 12 h. The obtained optical density (OD) values were plotted as seen in 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 optical density OD = 0.5 (time 0 h). Then bacteria were washed three times with Mueller–Hinton media with antibiotics and glucose to switch off antitoxin expression and were incubated for 4 h at 37 °C. Total bacteria were calculated by the c.f.u. per ml at time 0 h and 4 h present in Mueller–Hinton 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 37 °C for 2–3 h. 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 4 h at 37 °C on filter in Mueller–Hinton plates supplemented with DAP (and containing NaCl until 332 mM final concentration in the case of *V. vulnificus*).

In vivo conjugation in zebrafish larvae and *A. 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 number A-75–1061. Conjugative killing was assessed as follow. Four-day post-fertilization zebrafish larvae were exposed to water containing 10^4 c.f.u. per ml of *V. cholerae* O139 for 2 h at 27 °C (Fig. 4a) or a 1:1 mixed population containing 10^5 c.f.u. per ml *V. cholerae* O139 + *V. cholerae* O1 (mix *Vibrio*) for 2 h at 27 °C. Then, larvae were washed in sterile water three times and then placed into a well containing 10^7 or 10^6 c.f.u. per ml (Fig. 4a, *V. cholerae* O139 and mix *Vibrio*, respectively) of the *E. coli* β3914-*Δdap* donor strain containing either the pN_{ctrl} or pFW plasmid for 24 h at 27 °C. In Supplementary Fig. 11b,c, 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 no. E10521) at 200 mg ml^{-1} to euthanize them. Finally, they were transferred to a tube containing calibrated glass beads (acid washed, 425–600 µm, Sigma-Aldrich no. G8722) and 500 µl of water. Five larvae were mashed using FastPrep Cell Disrupter (BIO101/FP120 QBioGene) for 45 s at maximum speed (6.5 m s^{-1}) to analyze their microbiota (Supplementary Fig. 11) in Mueller–Hinton Media + X-gal or thiosulfate-citrate-bile salt-sucrose (TCBS) media for selection of *V. cholerae*. Blue bacteria corresponding to *V. cholerae* O139 were detected in Mueller–Hinton media. Transconjugants selection was done into Mueller–Hinton media + X-gal and Sp and, then, replication of these Mueller–Hinton plates was done on TCBS media to specifically 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 *A. salina* stage nauplii suspended in 1 ml volume of seawater were washed using sterile cell strainer Nylon filters with a 100 µm pore size (Falcon) and three times with the same volume (3 × 1 ml) of sterile PBS (D8537, Sigma). Nauplii were suspended in 1 ml PBS and then infected with 10^7 *V. cholerae* O1 or a mix of 10^7 *V. cholerae* O1 and O139 for 2 h in agitation at 27 °C. Then nauplii were washed as previously described and exposed to 10^7 of β3914-*Δdap* bacteria with pN_{ctrl} or pFW plasmid for 4 h at 27 °C. These experiments were repeated four times independently. The microbiota from 1 ml 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 fastprep (FastPrep Cell Disrupter (BIO101/FP120 QBioGene) for 45 s at maximum speed (6.5 m s^{-1})). Transconjugants were selected from 225 ± 15 nauplii after pN_{ctrl} or pFW conjugation treatment into Mueller–Hinton 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 Mueller–Hinton plates was done into TCBS medium to specifically identify *V. cholerae*. Strain identity was confirmed through yellow color development in TCBS *Vibrio*-specific medium.

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 follows. Four-day post-fertilization zebrafish larvae were exposed to water containing 10^6 c.f.u. per ml *V. cholerae O1*-GFP for 2 h at 27 °C. They were then washed in sterile water three times and placed into a well containing 10^7 c.f.u. per ml of *E. coli*-RFP for 24 h at 27 °C. Larvae were removed from the well and then placed into another well containing Tricaine to euthanize them. Infected and non-infected larvae were visualized by fluorescence microscopy (EVOS FL microscope, Life Technologies) using appropriate wavelength conditions to enable the visualization of GFP and RFP or not. Fluorescence was only detected in infected larvae and more precisely in 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 h in agitation at 27 °C. Then nauplii were washed as previously described and exposed to 10^7 of *E. coli*-RFP strain for 2 h. Microscopy conditions were performed as for the zebrafish experiment.

Statistics. In Supplementary Fig. 9, one-way analysis of variance with Dunnett's Multiple Comparison Test was performed. PNcontrol-R6K versus pPW-R6K, mean difference = 2.383×10^8, $q = 4.183$, $^{**}P < 0.05$, 95% confidence interval of difference = (8.937×10^7 to 3.871×10^8). PNcontrol-R6K versus pFW-R6K, mean difference = 2.308×10^8, $q = 4.227$, $^{**}P < 0.05$, 95% confidence interval of difference = (9.187×10^7 to 3.896×10^8).

In Supplementary Fig. 13, a one-sided *t*-test Mann-Whitney was performed. *E. coli* SXT versus *E. coli* MG1655. $P = 0.0143$. $^{*}P < 0.05$.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

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

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Experimental design

1. Sample size

   Describe how sample size was determined.

   For zebrafish and artemia models, we followed protocols and sample sizes from previously published studies

2. Data exclusions

   Describe any data exclusions.

   No data were excluded from the analyses.

3. Replication

   Describe the measures taken to verify the reproducibility of the experimental findings.

   Data (pictures and graphics) are representative of at least three independent experiments with similar results. Figure 4a (mix of Vibrios) represents the mean of two independent experiments using 10 zebrafish larvae. Independent experiments mean biological replicates. All attempts at replication were successful.

4. Randomization

   Describe how samples/organisms/participants were allocated into experimental groups.

   N/A

5. Blinding

   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   Blinding was not relevant to our study, as we follow the physical transfer of plasmids among bacteria, we have internal control in each assay.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

6. Statistical parameters

   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

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☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
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Materials and reagents

Policy information about availability of materials

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Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.	There are no restriction on availablity of our unique materials. All reagents are commercialy available.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	Not antibodies used

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	No eukaryotic cell lines were used
b. Describe the method of cell line authentication used.	No eukaryotic cell lines were used
c. Report whether the cell lines were tested for mycoplasma contamination.	No eukaryotic cell lines were used
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	No eukaryotic cell lines were used

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.	Four-day post-fertilization zebrafish (Danio rerio) larvae. Artemia salina stage nauplii.

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Describe the covariate-relevant population characteristics of the human research participants.	This study did not involve human research participants.