



Conjugation Inhibitors Effectively Prevent Plasmid Transmission in Natural Environments

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ABSTRACT Plasmid conjugation is a major route for the spread of antibiotic resistance genes. Inhibiting conjugation has been proposed as a feasible strategy to stop or delay the propagation of antibiotic resistance genes. Several compounds have been shown to be conjugation inhibitors *in vitro*, specifically targeting the plasmid horizontal transfer machinery. However, the *in vivo* efficiency and the applicability of these compounds to clinical and environmental settings remained untested. Here we show that the synthetic fatty acid 2-hexadecynoic acid (2-HDA), when used as a fish food supplement, lowers the conjugation frequency of model plasmids up to 10-fold in controlled water microcosms. When added to the food for mice, 2-HDA diminished the conjugation efficiency 50-fold in controlled plasmid transfer assays carried out in the mouse gut. These results demonstrate the *in vivo* efficiency of conjugation inhibitors, paving the way for their potential application in clinical and environmental settings.

IMPORTANCE The spread of antibiotic resistance is considered one of the major threats for global health in the immediate future. A key reason for the speed at which antibiotic resistance spread is the ability of bacteria to share genes with each other. Antibiotic resistance genes harbored in plasmids can be easily transferred to commensal and pathogenic bacteria through a process known as bacterial conjugation. Blocking conjugation is thus a potentially useful strategy to curtail the propagation of antibiotic resistance. Conjugation inhibitors (COINS) are a series of compounds that block conjugation *in vitro*. Here we show that COINS efficiently block plasmid transmission in two controlled natural environments, water microcosms and the mouse gut. These observations indicate that COIN therapy can be used to prevent the spread of antibiotic resistance.

KEYWORDS *Escherichia coli*, bacterial conjugation, antibiotic resistance, conjugation inhibitor, microcosm, gut, zebrafish, mouse, conjugation, plasmids

The spread of antibiotic resistance genes (ARGs) among bacterial populations is one of the major threats in global health care. According to existing estimates, approximately 700,000 people die annually because of infections caused by resistant bacteria (1, 2), a figure likely to increase, as the number of strains resistant to various antibiotics is on the rise in a wide range of pathogenic species (3). ARGs may emerge as vertically inherited chromosomal mutations or may be encoded by mobile genetic elements (MGEs) able to spread both vertically and horizontally among bacteria. Among MGEs, conjugative plasmids are of special relevance in the spread of ARGs (4). Conjugative plasmids exhibit a wide host range and thus are able to shuttle ARGs between different genera, orders, and even phyla (5). Moreover, when they invade a new host, they trigger the SOS response, driving the swapping of integron cassettes and promoting the generation of multiresistant

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strains (6). Preventing plasmid conjugation is therefore a key step in curtailing the propagation of ARGs, and a variety of strategies have been proposed (7, 8). Conjugation inhibitors (COINs) are compounds that inhibit plasmid transfer by affecting the conjugative machinery. Unsaturated fatty acids are COINs blocking the transfer of various groups of plasmids *in vitro* (9 to 11). However, their *in vivo* efficiency remained questioned for two major reasons: they require relatively high concentrations to efficiently prevent conjugation, and they can be metabolized by cells as an energy source. On the other hand, synthetic derivatives such as 2-hexadecynoic acid (2-HDA) (C₁₆H₂₈O₂) were shown to be more chemically and biologically stable (9). 2-HDA is a potent COIN, able to block the plasmid secretion machinery at low concentrations (12) and prevent conjugation in a variety of plasmids and host species (9), yet its *in vivo* efficiency was not assessed.

We thus decided to test the ability of 2-HDA to block conjugation in two *in vivo* models: a freshwater microcosm and the mouse gut. Water environments are considered hot spots for the propagation of antibiotic resistance, due to the selective pressure exerted by residual antibiotic concentrations present in water effluents (13, 14). The gut is another important niche in the transmission of antibiotic resistance. The gut microbiota acts as a reservoir of ARGs that may be transferred to pathogens (15), and plasmid conjugation has been demonstrated in murine models (16–18) and in the fish gastrointestinal tract (19).

Conjugation experiments were carried out between spontaneous nalidixic (Nx)- and rifampicin (Rif)-resistant derivatives of *Escherichia coli* MDS42 (20). Conjugation frequencies were determined by plating in appropriate selective antibiotics (21). The water microcosms consisted of three 36-liter aquaria, kept at 28°C and equipped with continuous water flow systems and appropriate filters for water homeostasis, as described in Text S1 in the supplemental material. Aquaria were inhabited by three fish species, *Hypostomus plecostomus* (suckermouth catfish), *Corydoras paleatus* (peppered cory catfish), and *Danio rerio* (zebrafish). Additionally, the microcosms included two species of grass, *Vallisneria gigantea* (eelgrass) and *Lemna minor* (common duckweed), and spontaneously acquired invertebrates. We used 16S metataxonomic analyses to characterize the native bacterial population of the aquarium and search for possible species (*Enterobacteriaceae* or other *E. coli* strains) that could act as conjugation recipients for our experiments (see Fig. S1 in the supplemental material). As described in the supplemental material, neither native aquarium strains nor wild-type *E. coli* isolated from other aquatic environments (Table S1) behaved better than laboratory strains (Fig. S2 and S3). We thus selected *E. coli* MDS42 and MDS52 as our recipient strains and checked that 2-HDA did not inhibit or delay bacterial growth (Fig. S4).

As shown in Fig. 1A, the conjugation experiments were performed by soaking fish food pellets with donor and recipient bacterial cultures, thus obtaining donor and recipient pellets. These pellets were then added as food to the fish inside a breeding box, installed into the fish tank, to facilitate feeding and the collection of fecal samples. Experiments were performed with and without fishes inside the breeding box, as controls. As shown in Fig. 1B, after 24 h, we retrieved more than 10⁶ CFU per mg of food, when inoculation was performed in empty breeding boxes. When a fish was present in the breeding box, the number of viable counts reached 10⁷ CFU per mg of fecal matter. The results thus demonstrated that the inoculation procedure achieved sufficient cell densities and that bacterial transit through the fish allowed the *E. coli* viable counts to increase. We calculated conjugation frequencies (CF) using two model plasmids, pOX38, from the PTU-F_E group and R388 from the PTU-W group. Results are shown in Fig. 1B. In the absence of fish, conjugation occurred at low frequencies of approximately 10⁻⁶ transconjugants (T) per donor (D) cell. However, when fishes were present, the CF reached 10⁻⁴ T/D. These results indicated that transit through the fish gut increased the conjugation frequency of the plasmids.

We then assessed the effect of 2-HDA on the CF. When conjugation was performed *in vitro*, in a growth medium composed of filtered tank water enriched with 10% LB,

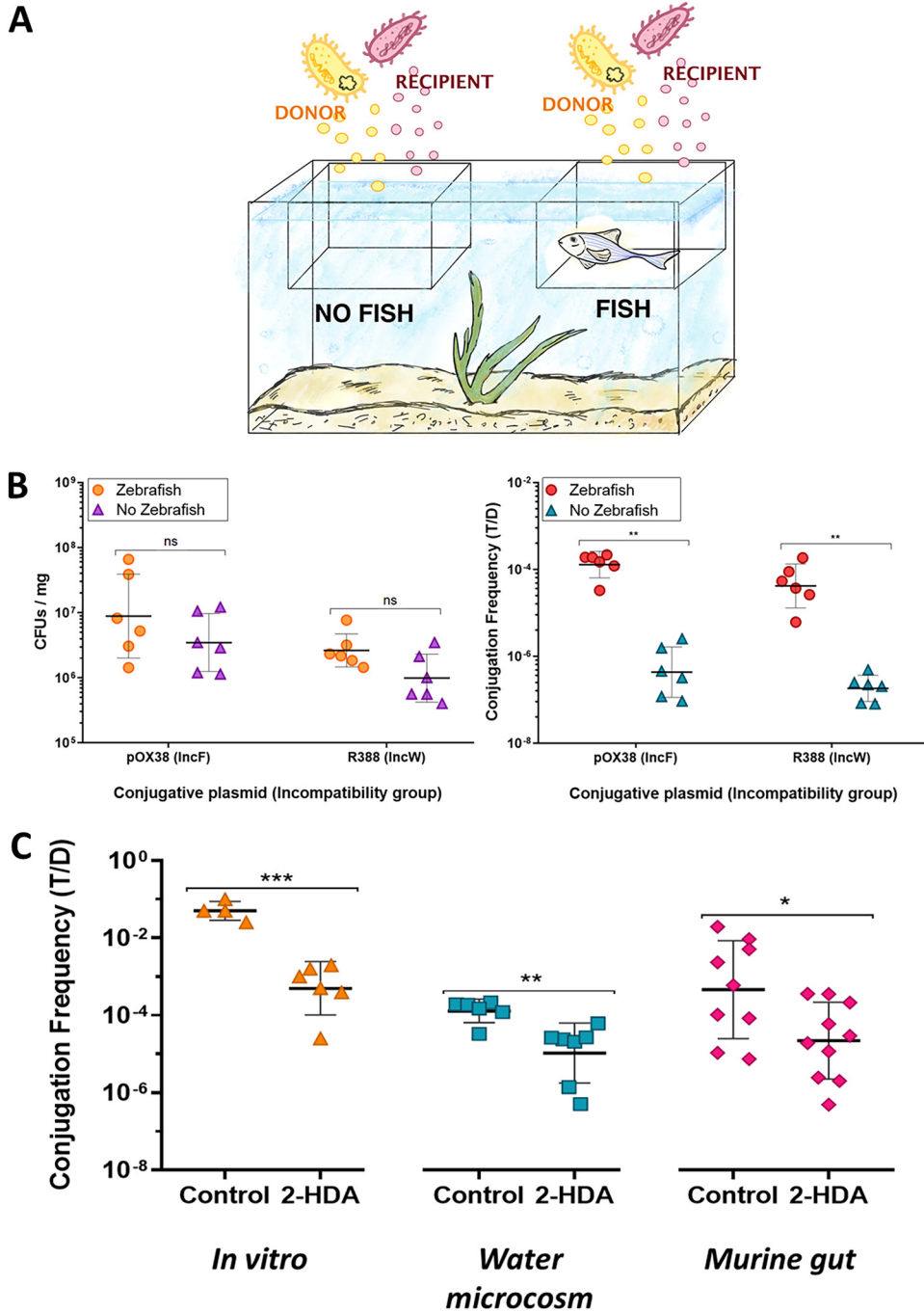


FIG 1 (A) Scheme depicting the administration strategy in water microcosms. Protocol specifications are shown in Text S1 in the supplemental material. (B) *E. coli* MDS42 survival (left) and conjugation rate (right) in the water microcosms. On the x axis are indicated the plasmids carried by the donor bacteria. Each symbol represents the value for an independent experiment. The horizontal bars show the experimental group average. Statistical significance of the average differences, as inferred from a *t* test are shown by asterisks (**, $P < 0.01$; ns, not significant). (C) Effect of 2-HDA administration on the CF of plasmid pOX-38 in different settings. On the x axis are indicated the experimental and control groups, while on the y axis is the CF expressed as number of transconjugants (T) per donor bacteria (D). Each symbol represents the value for an independent experiment. The horizontal bars show the experimental group average. Statistical significance of the average differences, as inferred from a *t* test are shown by asterisks (*, $P < 0.05$; **, $P < 0.01$).

the CF of plasmid pOX38 reached more than 10^{-2} T/D. This figure was reduced 50-fold when a final concentration of $100 \mu\text{g/ml}$ 2-HDA was added to the medium. Similarly, when 2-HDA was added to the food at a concentration of $1.6 \mu\text{g/mg}$ and fed to fish, the CF measured in the fecal pellets decreased >10 -fold (Fig. 1C). These results showed that administration of 2-HDA in the food efficiently decreased the ability of plasmids to propagate through conjugation, without inhibiting the bacterial growth rate (Fig. S4).

In order to test whether these results were contingent on the model system, we repeated the experiment in a second setting: the mouse gut. For this purpose, C57BL/6 mice were inoculated with donors and recipients through oral gavage, and the presence of transconjugants in the fecal pellets was evaluated 24 h later, as detailed in Text S1. The presence of confounding Nx^r or Rif^r *E. coli* strains in the mice microbiota was ruled out prior to the experiment, as described in Text S1. A dose of 10^9 cells of donor and recipient bacteria were inoculated to the mice, and supplemented with $100 \mu\text{g}$ of 2-HDA in the case of the experimental group. When donors and transconjugants from fecal pellets were enumerated, a 10-fold decrease in the CF was observed for the group treated with the COIN. The results thus demonstrated that 2-HDA achieved nearly 90% efficiency in blocking plasmid conjugation in both microcosms (freshwater and mouse gut). Although toxicity was not specifically tested in the experimental setup, no apparent toxic effects were observed in either the fish or the mice during the course of the experiments. Further research is required to better assess potential 2-HDA side effects, as well as determining its impact on the resident microbiota. Altogether, results shown here demonstrate that administration of a prototype COIN into clinical and environmental settings is a feasible strategy to prevent the transmission of antibiotic resistance. Because the persistence of a conjugative plasmid is a delicate balance between the ability to propagate and the burden imposed on the host, a 10-fold decrease in the CF may lead the plasmid to extinction, as demonstrated by previous results (9). Further research is thus required to determine whether COINS can be used *in vivo*, not only to block propagation but also to purge the microbiota from ARG-containing plasmids. This would open the possibility of using COINS as adjuvants to antibiotic treatment in order to prevent the rise and persistence of antibiotic-resistant bacteria.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOCX file, 0.02 MB.

FIG S1, TIF file, 0.9 MB.

FIG S2, TIF file, 0.3 MB.

FIG S3, TIF file, 0.2 MB.

FIG S4, TIF file, 0.2 MB.

TABLE S1, DOCX file, 0.1 MB.

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