

Synthetic Fatty Acids Prevent Plasmid-Mediated Horizontal Gene Transfer

María Getino,^a David J. Sanabria-Ríos,^b Raúl Fernández-López,^a Javier Campos-Gómez,^{a*} José M. Sánchez-López,^c Antonio Fernández,^c Néstor M. Carballeira,^d Fernando de la Cruz^a

Instituto de Biomedicina y Biotecnología de Cantabria, Universidad de Cantabria—Consejo Superior de Investigaciones Científicas, Santander, Cantabria, Spain^a; Faculty of Science and Technology, Interamerican University of Puerto Rico, Metropolitan Campus, San Juan, Puerto Rico^b; Biomar Microbial Technologies, Parque Tecnológico de León, Armunia, León, Spain^c; Department of Chemistry, University of Puerto Rico, Rio Piedras Campus, San Juan, Puerto Rico^d

* Present address: Javier Campos-Gómez, Department of Molecular Biology and Biochemistry, Drug Discovery Division, Southern Research Institute, Birmingham, Alabama, USA.

ABSTRACT Bacterial conjugation constitutes a major horizontal gene transfer mechanism for the dissemination of antibiotic resistance genes among human pathogens. Antibiotic resistance spread could be halted or diminished by molecules that interfere with the conjugation process. In this work, synthetic 2-alkynoic fatty acids were identified as a novel class of conjugation inhibitors. Their chemical properties were investigated by using the prototype 2-hexadecynoic acid and its derivatives. Essential features of effective inhibitors were the carboxylic group, an optimal long aliphatic chain of 16 carbon atoms, and one unsaturation. Chemical modification of these groups led to inactive or less-active derivatives. Conjugation inhibitors were found to act on the donor cell, affecting a wide number of pathogenic bacterial hosts, including *Escherichia*, *Salmonella*, *Pseudomonas*, and *Acinetobacter* spp. Conjugation inhibitors were active in inhibiting transfer of IncF, IncW, and IncH plasmids, moderately active against IncI, IncL/M, and IncX plasmids, and inactive against IncP and IncN plasmids. Importantly, the use of 2-hexadecynoic acid avoided the spread of a derepressed IncF plasmid into a recipient population, demonstrating the feasibility of abolishing the dissemination of antimicrobial resistances by blocking bacterial conjugation.

IMPORTANCE Diseases caused by multidrug-resistant bacteria are taking an important toll with respect to human morbidity and mortality. The most relevant antibiotic resistance genes come to human pathogens carried by plasmids, mainly using conjugation as a transmission mechanism. Here, we identified and characterized a series of compounds that were active against several plasmid groups of clinical relevance, in a wide variety of bacterial hosts. These inhibitors might be used for fighting antibiotic-resistance dissemination by inhibiting conjugation. Potential inhibitors could be used in specific settings (e.g., farm, fish factory, or even clinical settings) to investigate their effect in the eradication of undesired resistances.

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Address correspondence to Fernando de la Cruz, delacruz@unican.es.

Infections due to enterobacteria carrying antibiotic resistance (AbR) determinants are a major cause of global morbidity and mortality (1). Despite their ongoing success, antibiotics are becoming a progressively more limited weapon to fight bacterial infections. Over the past years, few novel antibiotics have been developed and larger numbers of pathogens resistant to current treatments have arisen (2). Since AbR mechanisms are naturally present in antibiotic-producing organisms, they can easily spread to bacterial pathogens by horizontal gene transfer (HGT). In enterobacteria, plasmid conjugation is one of the main sources of HGT, and the emergence of multiresistant pathogens is frequently linked to the spread of conjugative plasmids. For example, worldwide dissemination of extended-spectrum beta-lactamases, particularly the CTX-M enzymes, is due to mobile genetic elements, especially conjugative plasmids from the IncF group (3). Because AbR genes disseminate mostly by conjugation, strategies to control conjugation could provide effective means to curb AbR dissemination (4, 5). Among the proposed alternatives to conven-

tional antibiotics, this work focuses on the development of chemical inhibitors of bacterial conjugation.

Previous efforts to control conjugation in enterobacteria focused on two complementary lines of action. The first line of action consisted of chemical and biological agents acting against key molecular components of the conjugation process. One of those key components is the relaxase, the protein responsible for nicking DNA at the origin of transfer and initiating plasmid transfer. Relaxase activity was inhibited by the use of bisphosphonates (6), a strategy whose results were later revealed to be misleading, since these compounds were found to act as unspecific chelating agents (7). Another strategy involved the expression of intrabodies directed against plasmid R388 relaxase. Intrabodies were expressed in recipient cells, successfully preventing the acquisition of the conjugative plasmid (8). However, the applicability of intrabodies in clinical or environmental settings is limited, since it requires a transgenic recipient population expressing the intrabody. Another specific target for the control of conjugation was the conju-

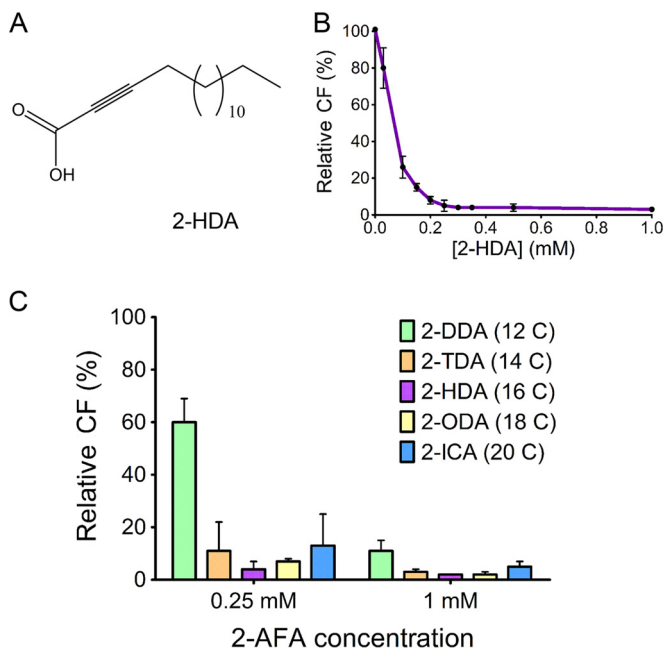


FIG 1 2-Hexadecynoic (2-HDA) COIN activity. (A) Chemical structure of 2-HDA. (B) Conjugation frequencies (CF) in the presence of increasing concentrations of 2-HDA relative to CF in the absence of 2-HDA (100%). Values represent means \pm standard deviations (SD) of the results of at least five independent experiments measured by HTC assay. (C) CF in the presence of five 2-AFAs with different chain lengths (12 to 20 carbon atoms) at two different concentrations relative to CF in the absence of added compounds (100%). The values represent means \pm SD of the results of at least three independent experiments measured by HTC assay.

gative pilus. Certain bacteriophages attach to conjugative pili with high specificity. By exploiting the natural affinity of bacteriophage M13 for the F pilus, this bacteriophage and its protein, pIII, were employed to inhibit F plasmid conjugation (9). This strategy would be most useful if it could be extended to other types of pili.

A second line of action for developing conjugation inhibitors (COINs) involved whole-cell assays, i.e., screening for compounds that produce reduced numbers of transconjugant cells in conventional conjugation assays. This approach suffers from a major backlash: the possibility of false positives arising from compounds that do not target the conjugative machinery but inhibit cell growth instead. Indeed, many early compounds described as COINs were later found to be growth inhibitors (10–12). Using a luminescence-based high-throughput conjugation (HTC) assay, in combination with a secondary assay that ruled out effects on growth rates, unsaturated fatty acids (uFAs) were discovered as the first effective COINs. uFAs were found to inhibit conjugation of IncW and IncF plasmids, while cell growth was not affected (13). Screening a library of 12,000 natural compounds (NatChem library) yielded dehydrocrepenynic acid (DHCA) as the fatty acid with the highest COIN activity (13). However, DHCA has to be extracted from tropical plant seeds (14), complicating the characterization of its COIN activity. As a result, it was unclear whether DHCA and other uFAs were potent enough to efficiently block the spread of conjugative plasmids and the range of bacterial hosts susceptible to inhibition.

In this work, starting from the chemical structure of DHCA, we developed simple synthetic COINs in sufficient amounts to study

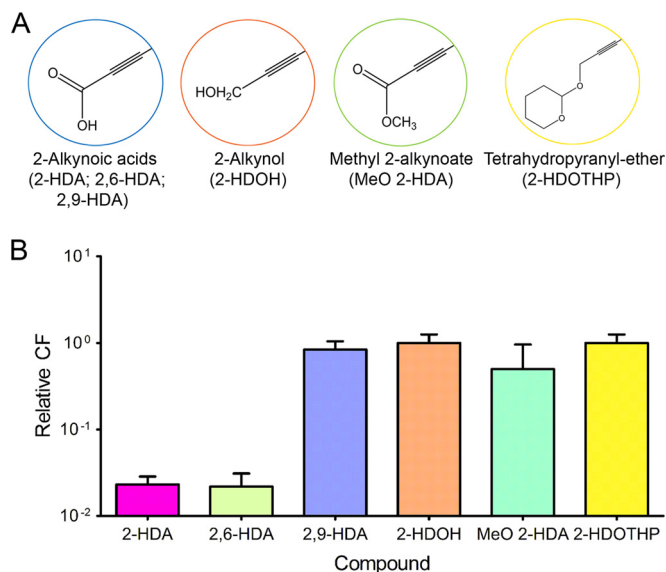


FIG 2 COIN activity of 2-HDA analogs. (A) Schematic structures of functional groups of analyzed compounds. 2-HDOH, 2-hexadecyn-1-ol; 2-HDOTHP, 2-(2-hexadecynoxy)-tetrahydro-2H-pyran. (B) Conjugation frequencies (CF) represented in logarithmic scale in the presence of 1 mM 2-HDA and related compounds relative to a control without added compounds (10^0). Values represent means \pm SD of the results of at least three independent experiments measured by HTC assay.

their efficiency and range of activity. We found that synthetic 2-hexadecynoic acid (2-HDA) acts as a true COIN on a wide range of bacterial species and conjugative plasmids. Importantly, 2-HDA was able to prevent the spread of the highly infective IncF plasmid R1drd19, thus demonstrating the feasibility of using COINs to block the spread of AbR.

RESULTS

2-HDA, an effective synthetic COIN. DHCA was identified in previous work (13) as the most potent COIN found. DHCA is one of the 2-alkynoic fatty acids (2-AFAs), a class of molecules known for their bioactive properties (15, 16). To test whether other 2-AFAs shared COIN activity with DHCA, we tested a number of them that were simpler and amenable to chemical synthesis (15–17). 2-HDA is a 2-AFA with a chain length of 16 carbon atoms and one triple bond at C-2 (Fig. 1A). In addition to its previously reported activities, 2-HDA inhibited R388 conjugation to 2% at 0.3 mM (98% inhibitory concentration [IC_{98}], 0.3 mM) (Fig. 1B). When five monounsaturated 2-AFAs of different chain lengths were compared to test for the influence of hydrocarbon chain length on COIN activity, 2-HDA showed an optimal chain length (Fig. 1C). As shown in Fig. 1C, COIN potency follows the trend 2-HDA (16 C) > 2-octadecynoic acid (2-ODA; 18 C) > 2-tetradecynoic acid (2-TDA; 14 C) > 2-icosynoic acid (2-ICA; 20 C) > 2-dodecynoic acid (2-DDA; 12 C).

The carboxylic group is essential to COIN activity. A set of chemical analogs of 2-HDA were synthesized to ascertain which chemical groups were crucial for the observed activity. The carboxylic group was substituted for other functional groups in the studied analogs, including 2-alkynols, methyl 2-alkynoates, and tetrahydropyranyl-ethers (Fig. 2A). Interestingly, only 2-HDA derivatives with an unaltered carboxylic group remained active

TABLE 1 Conjugation frequency in the presence of 2-HDA and 2-ODA

Plasmid	Inc ^b	MOB ^c	MPF ^d	CF (%) ^a						
				2-HDA (mM)			2-ODA (mM)			
				0.2	0.4	1	0.2	0.4	1	
R388	W	F11	T	3**	1***	1***	29	9**		
pSa	W	F11	T	—	1***	—	—	—		
pIE321	W	F11	T	—	1***	—	—	—		
pIE522	W	F11	T	—	1***	—	—	—		
R7K	W	F11	T	—	1***	—	—	—		
pMBUI4	W-like	F11	T	—	1***	—	—	—		
pKM101	N	F11	T	62	89	55	75	165		
pOX38	FI	F12	F	22*	5***	3***	71	10**		
R1drd19	FII	F12	F	11**	3***	3***	25*	11**		
R100-1	FII	F12	F	5***	1***	1***	16*	2***		
pRL443	P1 α	P11	T	122	99	80	137	154		
R751	P1 β	P11	T	117	55	154	57	55		
R64drd11	I1 α	P12	I	90	14*	4**	51	47		
pCTX-M3	L/M	P131	I	135	40	11**	180	51		
R6K	X ²	P3	T	47	27*	17*	57	26*		
drR27	H11	H11	F	9***	6***	3***	27**	15***		

^a Conjugation frequencies (CF) in the presence of 2-HDA and 2-ODA, determined using a representative set of conjugative plasmids, expressed as a percentage relative to a control without added COINs (100%). Values represent means of the results of at least four independent experiments measured by plate conjugation assay. *, $P < 0.05$ (mean significantly different from control); **, $P < 0.01$; ***, $P < 0.001$. The dashes represent the absence of data at the indicated concentrations.

^b Inc, incompatibility group (38).

^c MOB, MOB group (39).

^d MPF, mating pair formation type (40).

(Fig. 2B). The same behavior was observed with 2-ODA and its derivatives (see Fig. S1 in the supplemental material). Moreover, two 2-HDA derivatives containing two separate triple bonds, 2,6-hexadecadiynoic acid (2,6-HDA) and 2,9-hexadecadiynoic acid (2,9-HDA), were also assayed. Results are shown in Fig. 2. While 1 mM 2,6-HDA inhibited conjugation at the same level as 2-HDA, when the second triple bond was placed more distantly from the carboxylic group (2,9-HDA), no inhibition was observed (Fig. 2B). Unlike 2-HDA, 2,6-HDA was less active at lower concentrations. For instance, 2,6-HDA inhibits conjugation to only $30\% \pm 4\%$ at 0.3 mM. In summary, the combination of a long, unsaturated hydrocarbon chain plus a carboxylic group seems to be the outstanding chemical group required for COIN activity.

IncW, IncF, and IncH conjugative plasmids, the best targets.

Thus far, plasmid R388 was employed to test for COIN activity, which prompts investigation of the issue of how broad the range is of plasmids affected by the identified COINs. With the purpose of expanding this scope, a collection of prototype conjugative plasmids from *Enterobacteriaceae* (see Table S1 in the supplemental material) were tested with the most active 2-AFAs (2-HDA and 2-ODA). Results are shown in Table 1. As occurred with previously tested uFAs (13), conjugation of IncW and IncF plasmids was preferentially inhibited in the presence of 2-AFAs. Specifically, 2-HDA reduced by about 100 times the conjugation frequencies of IncW, IncF, and IncH plasmids at a concentration of 0.4 mM (Table 1). The conjugation characteristics of a number of MOB_{p11}/IncW plasmids such as pSa, pIE321, pIE522, R7K, and the IncW-like plasmid pMBUI4 were affected to the same extent as R388 itself (Table 1). When higher (1 mM) concentrations of 2-HDA were used, IncI, IncL/M, and IncX plasmids were also

TABLE 2 Mobilization frequency in the presence of 2-HDA and 2-ODA

Plasmid ^b	Inc ^c	MOB ^d	MPF ^e	MF (%) ^a						
				2-HDA (mM)			2-ODA (mM)			
				0.2	0.4	1	0.2	0.4	1	
CloDF13 (R388)	ColE	C11	-(T)	12**	9***	2***	29	26*		
ColE1 (pOX38)	ColE	P5	-(F)	8**	4***	3***	31*	11**		
ColE1 (pRL443)	ColE	P5	-(T)	100	77	70	100	87		
RSF1010 (pRL443)	Q1	Q11	-(T)	84	58	59	70	66		

^a Mobilization frequencies (MF) in the presence of 2-HDA and 2-ODA using three different mobilizable plasmids, expressed as a percentage relative to a control without added COINs (100%). Values represent means of the results of at least four independent experiments measured by plate conjugation assay. *, $P < 0.05$ (mean significantly different from control); **, $P < 0.01$; ***, $P < 0.001$.

^b The helper plasmids used are indicated in parentheses.

^c Inc, incompatibility group (38).

^d MOB, MOB group (39).

^e MPF, mating pair formation type (40). The minus signs represent the absence of MPF in mobilizable plasmids, which use helper MPF (in parentheses).

inhibited to various extents. Other plasmids (IncN and IncP) were not significantly affected even at the higher tested 2-HDA concentrations (Table 1). Hence, significant differences in the sensitivities of different plasmid conjugation systems to the tested COINs were observed, which could provide valuable insights regarding to their mode of action.

Effect of COINs on plasmid mobilization. In addition to conjugative plasmids, mobilizable plasmids are also transmissible by conjugation, when helped by a conjugative plasmid coexisting in the donor cell (18). Thus, it seemed interesting to test for the transfer of different mobilizable plasmids in the presence of diverse conjugative systems. This experiment would also help to elucidate the 2-AFA target in the conjugation machinery. Thus, several mobilizable plasmids were tested in the presence of either 2-HDA or 2-ODA, using different helper plasmids. As shown in Table 2, it was only when the helper plasmid was itself affected by COINs that conjugation of the mobilizable plasmid was inhibited. In contrast, when the ColE1 mobilizable plasmid was transferred using a COIN-resistant plasmid (IncP plasmid pRL443), its mobilization was not affected (Table 2). These results suggest a shared target of 2-AFAs in mobilization and conjugation, probably a part of the mating pair formation (MPF) system. An additional experiment to test this hypothesis was performed by mobilizing the *oriT*-MOB region of R388 (pHP161) (19) using the MPF system of plasmid pKM101 (20). When 0.4 mM 2-HDA was added to conjugation media, no inhibition effect was observed, as occurred for the transfer of plasmid pKM101 alone (see Fig. S2 in the supplemental material).

COINs act in a broad range of donor bacteria. Conjugation occurs when donor cells encounter recipient cells. However, which cells are the primary targets of the inhibition reaction? To answer this question, a modified conjugation inhibition assay was carried out. Donor or recipient cells were grown in the presence of 2-HDA, and conventional conjugation assays were performed in the absence of the COIN. Under these conditions, conjugation was inhibited only when donor cells were preincubated with

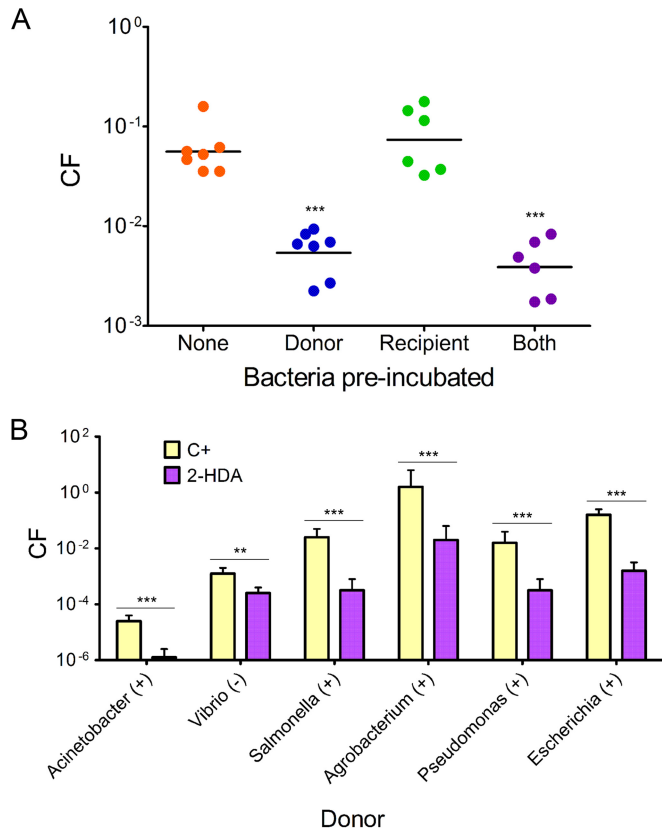


FIG 3 2-HDA effect in donor bacteria. (A) Conjugation frequencies (CF) of R388 shown in logarithmic scale after the donor or the recipient or both were grown overnight in the presence of 0.4 mM 2-HDA. Each point represents the result of one independent experiment measured by plate conjugation assay in the absence of COINs. Each horizontal bar represents the mean value of each group of data. ***, $P < 0.001$ (mean significantly different from control [orange]). (B) CF of R388 using different hosts as donor bacteria represented in logarithmic scale in the presence of 0.4 mM 2-HDA. C+, positive control in the absence of compound. The bars represent means \pm SD of the results of at least four independent experiments measured by plate conjugation assay. **, $P < 0.01$; ***, $P < 0.001$ (mean significantly different from control [yellow]). OD_{600} values for donor strains after a 24-h culture in the presence of 0.4 mM 2-HDA were similar to (+) or lower than (-) control values in the absence of the compound.

2-HDA, as shown in Fig. 3A. Preincubation of recipient cells did not show any effect. This simple experiment suggested that the tested COINs act on donor rather than on recipient cells. Therefore, with the purpose of finding out whether the observed COIN activity extends to other bacterial hosts besides *Escherichia coli*, various bacteria were analyzed as donors of plasmid R388. The plasmid was introduced into *Salmonella enterica*, *Acinetobacter baumannii*, *Vibrio cholerae*, *Agrobacterium tumefaciens*, and *Pseudomonas putida*, and these strains were used as donors in conventional mating experiments. R388 conjugation was inhibited in all five species in the presence of 2-HDA, as shown in Fig. 3B. In the case of *Vibrio cholerae*, the relative lack of effect seems to be due to inhibition of donor growth by 2-HDA (Fig. 3B). When plasmid pSLT, an indigenous IncFII plasmid from *S. enterica*, was tested in *S. enterica-E. coli* matings in both directions, its conjugation frequency also showed a significant reduction when the COIN was added to the mating medium (see Fig. S3 in the supplemental

material). These results imply that COINs are generally active in interspecies conjugation.

2-HDA suppresses R1drd19 spread. A key procedure regarding the feasibility of using COINs as an effective means to hinder AbR dissemination is to test their effect on the spread of a conjugative plasmid in a bacterial population that contains suitable receptor cells. Plasmid spread is conditioned by the burden plasmids impose on host cells. Because this burden results in lower growth rates, plasmid-free cells tend to outcompete plasmid-bearing cells. Plasmid-bearing cells, in turn, increase their numbers by conjugation. These two processes result in a dynamic situation where the fate of a plasmid depends on the equilibrium between plasmid infectivity and burden. This condition is classically known as the Steward-Levin equilibrium (21). We investigated whether 2-HDA was able to prevent the spread of the highly infective IncF plasmid R1drd19. Plasmid R1drd19 was chosen because of its ability to conjugate in liquid at high frequencies. This allowed us to monitor plasmid prevalence in a bacterial population that started with a 1:1 donor-to-recipient ratio (maximal transfer rate) and was allowed to grow for 15 generations. The proportion of plasmid-containing cells versus plasmid-free cells was determined at different time points by replica plating in populations that were subjected to different concentrations of 2-HDA (Materials and Methods). The results shown in Fig. 4A demonstrated that, in the absence of 2-HDA, plasmid R1drd19 quickly took over the population, with nearly 100% of the cells being positive for R1drd19 (R1drd19⁺) in 4 generations. In the presence of 400 μ M 2-HDA, however, the plasmid was unable to invade the population, and its prevalence slowly decayed from 50% to 27% during the course of the experiment.

To interpret these results, we built a simple ordinary differential equation model (equation 1) for determination of plasmid prevalence (see Text S2 in the supplemental material [supplemental calculations]) that includes frequency-dependent gains via conjugation and the effect of competition between plasmid-free and plasmid-containing cells in the absence of selective pressure for plasmid maintenance. Assuming a simple conjugation rate (γ) and a constant plasmid burden (b), the model predicts that plasmid spread depends on the magnitudes of γ and b as follows:

$$\bar{Y} = \frac{e^{(\gamma-b)t}}{e^{(\gamma-b)t} + \frac{\bar{Y}_0}{\bar{X}_0}} \quad (1)$$

where \bar{Y} stands for the proportion of plasmid-containing cells and \bar{Y}_0 and \bar{X}_0 indicate, respectively, the proportions of plasmid-containing and plasmid-free cells at time (t) 0. Exponential dependency results from the fact that transconjugants are also effective plasmid donors; thus, the proportion of plasmid-containing cells progresses geometrically. Results for different γ and b regimens are represented in Fig. 4B, showing situations where the plasmid progresses to invasion ($\gamma > b$), is driven to extinction by competition with plasmid-free cells ($\gamma < b$), or remains in equilibrium ($\gamma = b$).

To characterize the effect of 2-HDA on plasmid R1drd19 conjugation frequency (γ), we measured the burden (b) imposed by the plasmid (see Fig. S5 in the supplemental material). We then monitored plasmid progression at different 2-HDA concentrations, and, by fitting values to equation 1, we were able to extract

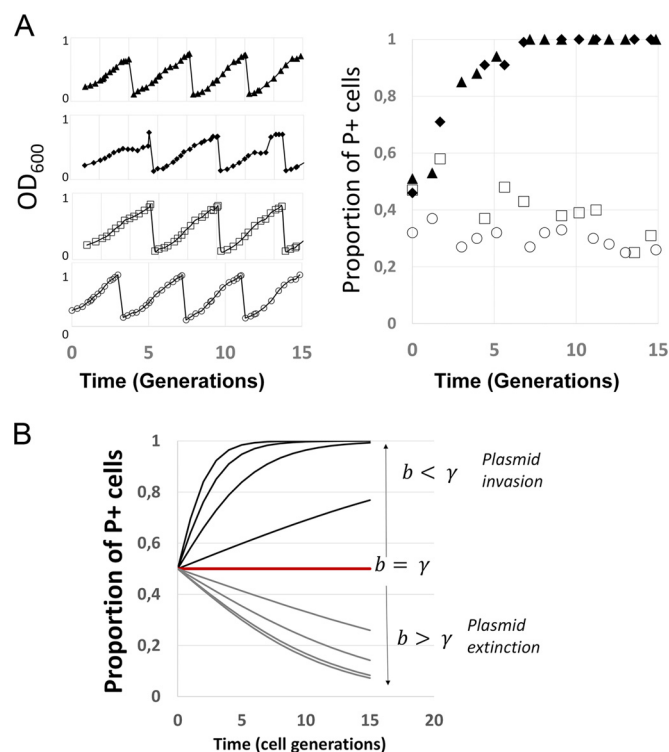


FIG 4 Effect of 2-HDA on plasmid R1drd19 spread in liquid medium. (A) Donor BW27783-Nx^r (R1drd19) cells and recipient BW27783-Rif^r cells, both in stationary phase, were mixed at a 1:1 ratio and diluted either in LB (solid diamonds and solid triangles) or in LB–0.4 mM 2-HDA (empty circles and empty squares) to a final OD₆₀₀ of 0.2. Cells were incubated at 37°C with constant agitation (80 rpm) in a turbidostatic regimen, OD₆₀₀ was monitored every 10 min (left panel), and when cells achieved an OD₆₀₀ of 0.8, they were diluted back to an OD₆₀₀ of 0.1 in LB (solid diamonds and solid triangles) or LB–0.4 mM 2-HDA (empty circles and empty squares). In each dilution cycle, samples were taken at OD₆₀₀s of 0.2, 0.4, and 0.8. Cells were diluted and plated in LB agar without antibiotics. From these plates, 100 colonies were replica plated on Km-containing plates to check for R1drd19 presence (right panel). The graph shows the proportion of R1drd19-containing cells in the population (number of Km^r cells/total number of cells replicated). (B) Conjugation frequency (γ) and burden on the host (b) determine plasmid fate. The graph shows the theoretical fractions of plasmid-containing cells through time (in cell generations) in a population of size n that contained equal numbers of plasmid-free (x) and plasmid-containing (y) cells at time 0. Plasmid-free cells multiply at rate α , while plasmid-containing cells suffer from plasmid-imposed burden b . Conjugation takes place at frequency γ . Under these assumptions, plasmid fate depends on the magnitudes of γ and b . In cases where $\gamma > b$, plasmid invasion progresses and eventually overtakes the entire population. In cases where $\gamma < b$, plasmid-containing cells are driven to extinction.

($r^2 > 0.95$) the apparent γ for each 2-HDA concentration used (Fig. 5A). A plot of the apparent γ values revealed a 50% inhibitory concentration (IC₅₀) of approximately 50 μ M 2-HDA (Fig. 5B), equivalent to the IC₅₀ observed in the dose-response assays for plasmid R388 conjugation (Fig. 1B). Overall results indicated that 2-HDA prevented the spread of highly infectious plasmid R1drd19 under conditions that maximize its transfer rate (exponential growth, 1:1 donor-to-recipient ratio). Moreover, given the burden imposed by the plasmid, in the absence of conjugation, the proportion of plasmid-containing cells decayed. This indicates that 2-HDA could be used not only to block plasmid transfer into susceptible cells but also to diminish plasmid prevalence in plasmid-containing populations.

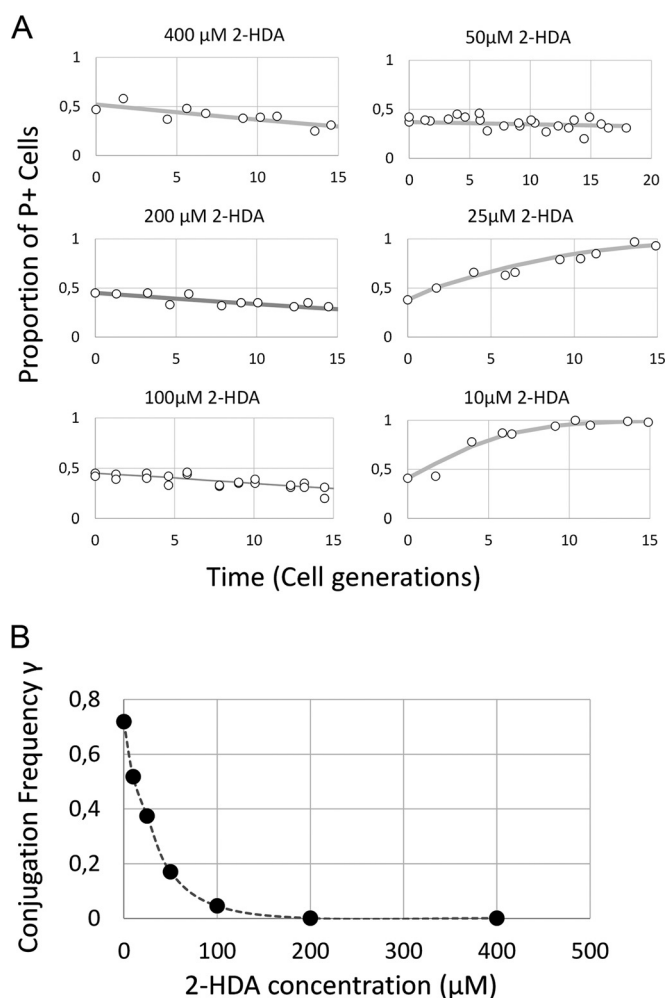


FIG 5 Prevention of plasmid spread is dose dependent. (A) Experimental result (open circles) and theoretical fit (grey lines) for assays of plasmid spread using different concentrations of 2-HDA. Experimental measurements were performed as described in the Fig. 4 legend, and the figure shows the proportions of plasmid-containing cells (x axis) through time (y axis, in cell generations). Nonlinear least-square fitting to equation 1 (grey lines) using the Levenberg-Marquardt algorithm was employed to determine the apparent γ values for each 2-HDA concentration used. (B) Apparent γ values (x axis) were plotted against the corresponding 2-HDA concentrations (y axis) to determine the dose-response curve of 2-HDA in plasmid R1drd19 spread. Results yielded an IC₅₀ of approximately 50 μ M.

DISCUSSION

The fast spread of AbRs demands effective means to stop, or at least slow down, their dissemination. Whole-cell analysis demonstrated that uFAs are efficient inhibitors of bacterial conjugation (13). However, uFAs obtained from natural sources presented a number of limitations that prevented the characterization of their COIN activity. Stable uFAs with potent COIN activity, such as DHCA, were difficult to obtain from natural sources. Other available uFAs, such as oleic acid and linoleic acid, presented lower COIN activity or were highly unstable due to auto-oxidation (22). Progress in COIN development required inhibitors that were stable and easily obtainable by chemical synthesis.

We have shown in this work that 2-AFAs, simple uFAs that can be synthesized chemically (15–17), possess COIN activity. Among

them, 2-HDA was the most potent COIN, with an IC_{98} of 0.3 mM (Fig. 1B), similar to that of natural uFAs (13). As in the case of natural uFAs, 2-HDA inhibited conjugation without disrupting cell growth (Fig. 3B), and 2-HDA was effective against the same range of plasmids (Table 1) (13). Taken together, the data indicate that synthetic 2-AFAs are suitable substitutes for natural uFAs. Because of their synthetic nature, we were able to test the relative levels of importance of different parts of the molecule in its COIN activity. The presence of a carboxylic group and one unsaturation proved to be essential features for COIN activity (Fig. 2; see also Fig. S1 in the supplemental material) (13). The length of the carbon chain was also important, with 16 carbons found to be the optimal length for the aliphatic chain (Fig. 1C). The presence of other triple bonds did not increase COIN activity (2,6-HDA) or even abolished it (2,9-HDA) (Fig. 2B). 2-AFAs are bioactive compounds, with antifungal and even antibacterial activity against certain species (although not against *E. coli* or the other species tested in this study except, perhaps, *V. cholerae*). Importantly, most of their bioactive properties display a similar dependency on the chemical features that correlate with potent COIN activity. Antifungal (23), antiprotozoal (15), and antibacterial (16) activities are higher for 2-HDA and 2-ODA, the 2-AFAs that displayed the higher COIN activity (Fig. 1C).

In addition to potency and structural similarities, the shared spectra of action among the discovered COINs (13) (Tables 1 and 2) suggest a common mechanism of inhibition. A general metabolic disturbance of the bacterial cells could be invoked as the cause of inhibition. In favor of this alternative is the fact that high inhibitor concentrations are needed. At these concentrations, COINs might affect overall properties (e.g., fluidity, permeability, structural changes, etc.) of bacterial membranes and, as a consequence, affect the function of a number of membrane proteins (including conjugation proteins). However, two sets of results argue against this alternative. First, certain plasmids but not others were affected by these compounds (Table 1). Second, conjugation is inhibited irrespectively of the bacterial host, among a variety of donors used in different experiments (Fig. 3B; see also Fig. S3 in the supplemental material). Therefore, COINs seem to target the conjugation machinery directly. Indeed, inhibition of R388 conjugation after donor preincubation with 2-HDA (Fig. 3A) suggests a specific target in the donor cell. Identification of the particular range of plasmids affected could provide valuable insights regarding their mode of action, attending to differences between them. In this respect, it is significant that uFAs affect the function of proteins associated with the bacterial membrane (24–27), many of them being ATPases. Since R388 conjugation requires the active participation of at least five ATPases (TrwB, TrwC, TrwD, TrwK, and StbB) (28), it is possible that uFAs specifically interact with one or several of these proteins. In fact, preliminary biochemical data from our laboratory indicate that the traffic ATPase TrwD, a component of R388 MPF system, is inhibited by linoleic acid (29). Plasmids containing close homologs of TrwD (IncW group and related plasmids in Fig. S4) were also affected by these COINs. On the other hand, plasmid pKM101, which carries a TrwD homolog (TraG) incapable of replacing TrwD for R388 transfer (30), was not affected, as shown in Table 1. These data are consistent with the fact that plasmids mobilized by the affected conjugative plasmids, the main targets of these COINs, are also inhibited (Table 2), since they used the MPF system of their helper plasmid for mobilization. The absence of inhibition in a system where the

oriT-MOB of R388 (pHP161) (19) was mobilized by the MPF apparatus of pKM101 (20) (see Fig. S2) also reinforces this hypothesis.

In summary, 2-AFAs provide an important scaffold structure as a starting point in the search for optimal COINs. First, their simple structures and ease of synthesis provided reaction amounts sufficient to analyze the key chemical features of COINs (Fig. 2; see also Fig. S1 in the supplemental material). Second, 2-AFAs shared a relatively broad range of plasmids affected (Table 1), among them IncF plasmids, the most common AbR carriers in pathogenic *Enterobacteriaceae*, labeled as representing high risk in clinical settings (31). Third, analysis of the mode of action of 2-AFAs has revealed donors as the target cells where blockage can be installed (Fig. 3A) and MPF as the conjugative part affected (Table 2; see also Fig. S2), important advances into the search of the molecular target. Fourth, study of 2-HDA applicability has demonstrated that conjugation can be blocked in different hosts (Fig. 3B; see also Fig. S3) and led to the fundamental conclusion that the observed inhibition level is sufficient to prevent invasion of AbR-carrying plasmids into a bacterial population and even to reduce the total number of carrier cells (Fig. 4).

This last point is of special relevance for assessing potential applications of COINs. Although 2-HDA did not abolish conjugation at 100%, its effect on plasmid transfer was sufficient to reverse the Steward-Levin equilibrium from plasmid invasion to plasmid loss (Fig. 4). This indicates that even if some cells escape inhibition, the overall effect on the population is enough to prevent plasmid spread in the absence of selective pressure for plasmid maintenance. Moreover, because of the deleterious effect of plasmid burden on host fitness, COINs could be used to purge bacterial populations from transmissible plasmids. It is often observed with infectious agents that the imposed burden increases with transmissibility, with highly infective agents usually being more virulent than mildly infective ones (32). In the case of IncF conjugative plasmids, this phenomenon is well documented (33). Thus, COIN action decreases the risk of AbR spread through conjugation while exerting a selective pressure against highly transmissible plasmids. In this regard, COINs beg to be tested in specific environments (e.g., farms, fish factories, or, later, even clinical settings). The dynamics of target populations should be evaluated with COINs in the presence or absence of antibiotics to gain a firmer knowledge of their potential therapeutic utility.

MATERIALS AND METHODS

Construction of pJC01. Conjugative plasmid pJC01 was constructed by inserting a *gfpmut2* gene into an R388 plasmid (34) as described in Text S1 in the supplemental material (supplemental Materials and Methods).

Synthesis of 2-AFAs and analogs. The synthesis of 2-DDA, 2-TDA, 2-HDA, 2-ODA, and 2-ICA followed an already-published procedure (15–17). 2,6-HDA and 2,9-HDA were prepared as previously described (35). 2-Alkynols, methyl-ester, and tetrahydropyranyl-ether derivatives of 2-HDA and 2-ODA were synthesized as shown in the literature (16, 36).

HTC assay. A whole-cell automated assay for conjugation, based on fluorescence emission in transconjugants cells, was carried out in a Biomek 3000 liquid-handling robot (Beckman Coulter). Donor and recipient strains were grown to the stationary phase in LB broth with appropriate antibiotics. For surface conjugation experiments, donor and recipient cells were concentrated 4-fold and mixed at a 1:1 ratio. After that, 10 μ l of each resulting conjugation mixture was spotted onto 96-well microtiter plates (Bioster), previously prepared by adding 150 μ l LB 1% agar with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) and differ-

ent COINs. Mating plates were incubated at 37°C for 6 h to allow conjugation, that is, the transfer of pJC01 into the BL21 (DE3) recipient strain, where expression of T7 RNA polymerase induced by IPTG triggers green fluorescent protein (GFP) production (see Fig. S6 in the supplemental material). After this time, cells were resuspended in 200 μ l M9 broth, and 150 μ l of the suspension was transferred to a new plate. The optical density at 60 nm (OD₆₀₀) and GFP emission of the suspensions were measured in a Victor3 multilabel counter (PerkinElmer). Conjugation frequencies (CF) were estimated as the ratio of absolute fluorescence emitted by transconjugant cells to the OD₆₀₀ as a measurement of the total number of cells. Relative CF in the presence of a compound was thus determined as a fraction of the CF in the absence of it, adding the same volume of solvent.

Plate conjugation assay. For the plate-mating procedure, a 200- μ l mixture of equal volumes of previously washed donor and recipient cultures, with both in stationary phase, was centrifuged and resuspended in 15 μ l LB broth. Then, 5 μ l of this mixture was placed on top of 96-well microtiter plate wells containing 150 μ l LB agar (with or without COINs) and conjugation was allowed to proceed, in general, for 1 h at 37°C. In the case of pSLT, mating was performed for 4 h at 37°C. drR27 was allowed to conjugate for 2 h at 25°C. *A. tumefaciens* and *P. putida* matings were carried out for 1 h at 30°C. Bacteria were then resuspended in 150 μ l M9 broth, and corresponding dilutions were plated on selective media. Conjugation frequencies (CF) were calculated as the number of transconjugant cells per donor, whereas mobilization frequencies (MF) were calculated as the number of cells receiving the mobilizable plasmid per donor. Since frequency data of this type were log-normally distributed, means were calculated using decimal logarithms of data. The relative CF or MF in the presence of a compound was determined as a fraction of the CF or MF in the absence of it, adding the same volume of solvent.

R1drd19 liquid mating. BW27783-Nx^r (nalidixic acid resistance) donor cells containing R1drd19 (see Table S1) and BW27783-Rif^r (rifampin resistance) recipient cells, both in stationary phase, were mixed at a 1:1 ratio and diluted in either LB or LB–0.4 mM 2-HDA to a final OD₆₀₀ of 0.2. Cells were incubated at 37°C and 80 rpm. Samples were taken at an OD₆₀₀ of 0.2, 0.4, or 0.8, when cultures were diluted to an OD₆₀₀ of 0.1, allowing conjugation for three more generations (i.e., until an OD₆₀₀ of 0.8 was reached again). The dilution process was repeated three times. Individual samples were appropriately diluted and plated in LB agar without antibiotics. The resulting colonies were replica plated on kanamycin (Km)-containing plates to check for R1drd19 presence. The percentage of cells containing R1drd19 at each generation was calculated.

Statistical analysis. Mean comparison between two different conditions was carried out by using the *t* test tool from Prism (v 5.0) biostatistics software (GraphPad, San Diego, CA).

Phylogeny tree construction. A phylogeny tree of MOB_{F11} family relaxases was constructed by using the neighbor-joining tool of MEGA5 (37).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01032-15/-/DCSupplemental>.

- Figure S1, PDF file, 0.9 MB.
- Figure S2, PDF file, 0.1 MB.
- Figure S3, PDF file, 0.1 MB.
- Figure S4, PDF file, 0.1 MB.
- Figure S5, PDF file, 0.1 MB.
- Figure S6, PDF file, 0.1 MB.
- Table S1, PDF file, 0.05 MB.
- Text S1, PDF file, 0.1 MB.
- Text S2, PDF file, 0.1 MB.

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