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MINIREVIEW

Ecology and Evolution as Targets: the Need for Novel Eco-Evo Drugs and Strategies To Fight Antibiotic Resistance▽†

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In recent years, the explosive spread of antibiotic resistance determinants among pathogenic, commensal, and environmental bacteria has reached a global dimension. Classical measures trying to contain or slow locally the progress of antibiotic resistance in patients on the basis of better antibiotic prescribing policies have clearly become insufficient at the global level. Urgent measures are needed to directly confront the processes influencing antibiotic resistance pollution in the microbiosphere. Recent interdisciplinary research indicates that new eco-evo drugs and strategies, which take ecology and evolution into account, have a promising role in resistance prevention, decontamination, and the eventual restoration of antibiotic susceptibility. This minireview summarizes what is known and what should be further investigated to find drugs and strategies aiming to counteract the "four P's," penetration, promiscuity, plasticity, and persistence of rapidly spreading bacterial clones, mobile genetic elements, or resistance genes. The term "drug" is used in this eco-evo perspective as a tool to fight resistance that is able to prevent, cure, or decrease potential damage caused by antibiotic resistance, not necessarily only at the individual level (the patient) but also at the ecological and evolutionary levels. This view offers a wealth of research opportunities for science and technology and also represents a large adaptive challenge for regulatory agencies and public health officers. Eco-evo drugs and interventions constitute a new avenue for research that might influence not only antibiotic resistance but the maintenance of a healthy interaction between humans and microbial systems in a rapidly changing biosphere.

Insufficiency of current measures to control the emergence, selection, and spread of antimicrobial resistance. Antibiotic resistance (AbR) is widespread in nature, and the goal of eliminating all resistance genes is simply nonsense, as the natural function of most resistance genes has nothing to do with AbR (91). Most probably, there is a huge "intrinsic resistome" in bacterial organisms, composed of genes of varied phylogenetic origin that act as resistance genes only in the presence of the antibiotic (48, 60, 126). Cleansing nature of this gene pool is impossible. The most we can do is to try to control the emergence, selection, and spread of AbR genes in bacterial organisms interacting with humans, animals, or plants (158). The classical methods of controlling the emergence and spread of AbR are based on the discovery of new antimicrobial agents (mostly in genocentric research) (52, 158), reduction of chronic antibiotic-promoted bacterial mutagenic stress, recombination, and horizontal-transfer genetic events associated with low dosages (29, 82, 109, 143), suppression of phenotypic resistance (34, 119, 154), use of combinations of drugs (16, 28, 44), including antagonistic drug pairs (92, 140), early intensive (frontline) therapy, maintaining a low bacterial density (44, 47, 51), and more recently, surveillance of hypermutable organisms (85) and targeting controlling functions essential for infection (26, 32, 61, 112). Controlling selection of AbR is a major practical goal, which can be addressed again by the development of novel anti-infective drugs and the appropriate use of antibiotics, avoiding low dosages able to select low-level mutations that can also serve as stepping stones for high-level resistance (9, 10, 45). Major efforts have been made to reduce general overconsumption of antimicrobial agents and thus limit the exposure of eventual resistant variants of pathogenic and commensal bacteria to the high-intensity selective power of these agents (53). Finally, a classical approach to avoid the spread of AbR is based on general hygiene and containment (infection control) measures, decreasing contact between patients contaminated (infected or carriers) with resistant bacteria and noncontaminated patients (17, 18).

Unfortunately, these measures are becoming increasingly insufficient in the current global landscape of AbR (19, 106, 124). Avoiding the emergence of resistance in the individual patient is obviously important for the individual, but it has minimal effects in the community (123). The efficacy of classical methods of controlling selection and spread is inversely proportional to the density and penetration (discussed below) (33) of resistant organisms and their mobile genetic elements in particular environments. Measures that might be successful in early stages of the development of resistance or in hospitals or countries with low rates of AbR have no value in areas where resistance is already an established biological phenomenon (18, 115). Even in areas with low levels of AbR pollution,

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such as Sweden, recent studies have shown that a 2-year discontinuation of trimethoprim use had no influence at all on the *Escherichia coli* resistance rates (134). This was probably due to the widespread distribution of trimethoprim resistance genes (*dfr*) in replicons harboring other resistance determinants, thus ensuring coselection of *dfr* genes with other resistance genes (20). Some regions of the world are densely polluted with AbR, while others remain clean (63, 137). In a global world, sooner or later, resistance originating in these highly contaminated "sources of resistance" will invade environments that are still clean (137, 158). Some models suggest that there is a threshold for this phenomenon. One of these models shows that resistant organisms are constantly diluted and potentially extinguish in competition with constant immigration of susceptible bacteria in local environments, but such a trend might collapse due to the increase of resistant populations (16). Moreover, the success of resistant organisms contributes to the constant accumulation in the bacterial world of genetic platforms and vehicles able to efficiently recruit and spread novel resistance genes. AbR calls for more resistance and increases bacterial genetic evolvability in a phenomenon described as "genetic capitalism" (7). At this time, resistance might be reversible by current interventions only when it is rare; if resistance is frequent, reversibility is not to be expected.

Eco-evo strategies: a novel integrative approach. The eco-evo concept started to be discussed in the last several years (39, 103). The eco-evo concept can be defined as a perspective in which organisms are evaluated broadly in the light of evolution and ecology, rather than narrowly by the constraints of their behavior in the laboratory or in the clinical practice in relation to human infections (103). The concept was coined first to explain the shift in bacterial life histories from commensal to pathogenic organisms (39). This notion also applies to AbR. In the scope of this minireview, we apply the term eco-evo strategies for the strategies leading to interventions that aim not necessarily to kill resistant organisms but rather to prevent their emergence and evolution or to reestablish the antibiotic-susceptible populations. It should be pointed out that at this time, most of the possible eco-evo strategies remain highly speculative, and few of them have been tested in the field. However, they are frequently based on well-documented novel knowledge. At this time, data are accumulating about the population genetics not only of bacterial pathogenic and antibiotic-resistant species and clones but also of the mobile genetic elements (MGEs) transporting resistance genes and associated platforms, such as plasmids, transposons, or integrative conjugative elements (ICEs) (22, 55, 121, 122, 135, 157). We are also making continuous progress in the field of bacterial ecogenetics, a field of biological research that studies the role of genetic variation in response to variable environments (101) and in the field of pharmacogenomics combined with systems biology, bioinformatics, and patient treatment (32, 70, 100, 118, 158). The use of antibiotics not only selects for resistant bacteria, MGEs carrying resistance genes, and even particular sequences within these genes, but it also promotes the movement of genes and competence for genetic transformation (29, 30, 109). Indeed, antibiotics produce an effect that should be analyzed under the light of the multilevel selection theory, a new burst of neo-Darwinian approaches (146). Selection naturally changes the ecological context, as it modifies population ecology, which influences the whole evolution of microbial systems (5, 21). Therefore, not only resistant bacteria should be considered units of epidemiological surveillance of AbR, all other elements (pieces) able to shape the natural evolutionary history of AbR should also be considered (3, 121). Consequently, all these biological-genetic elements, at any level of the hierarchy, should become targets for intervention against AbR. Note that these genetic elements are frequently linked by cooperative interactions, so that altering the density of particular bacterial clones should have consequences on the selection and evolution of MGEs (74, 96, 110).

Such a perspective indicates the need and possibility of using "eco-evo" drugs, drugs acting not necessarily to cure the individual patient (although this might happen for some antivirulence eco-evo drugs) but to "cure" specific environments from AbR and to prevent or weaken the evolutionary possibilities (the evolvability) of the biological elements involved in AbR. In other words, this perspective proposes to combat (decontaminate, de-evolve) resistance not only in infected patients but in a whole population composed of infected and noninfected people alike, as occurs in hospitals, nurseries, or elderly care facilities, as well as in general hot environments ("resistance reactors"), facilitating the evolution of AbR (8, 159). By extension, other environments that can be successfully treated are farms, fish factories, and eventually water effluents. Indeed, the notion of an "ill environment" should be increasingly encouraged, and medical treatment-like approaches might be increasingly applied to prevent and cure biologically altered environments (6, 89, 114, 159).

General targets for eco-evo drugs and strategies. The ecology and evolution of AbR depend on the construction of interactive networks able to exchange resistance genes between organisms, and/or among MGEs and their platforms, as well as their intrinsic variability (3, 21, 62a, 96, 121, 149). Such variable networks ensure joint evolution at multiple levels, a kind of multilevel cooperation. The bases of such cooperation are connectivity and variability, the general target processes for eco-evo drugs. Expanding this view, at any level of the hierarchy (let us say, from clones to plasmids or genes), the ecology and evolution of antimicrobial resistance depend on the "four P's", the penetration, promiscuity, plasticity, and persistence of the different genetic units. The word "penetration" is used here, by analogy with the term used in security engineering, for elements having a high attack profile regarding existing networks (33) and refers to the ability of a genetic element from a particular system to enter and be present in other systems. Examples are bacterial clones able to efficiently colonize and spread in microbiomes of different hosts or broad-host-range plasmids disseminating in widely different bacteria. The term "promiscuity" refers to the abilities to not only enter and be present in other systems but to exchange genetic sequences with the members of the system. The word "plasticity" is applied here as tolerated variability in the genetic sequences, from changes in the sequence of nucleotides in a gene to changes in the order of genes in a genome (syntheny) or changes in the modular structure of genetic regions (modularity) or in the modulation of their expression. An example of this is the change in the order of gene cassettes within a particular integron. The word "persistence" is used for the ability of each one of the biological elements to "construct a

niche," so that permanent links with its surrounding environment (including the genetic context) are established, allowing long-term coexistence or fixation of the sequence or element in the system. In the following paragraphs, we review possible eco-evo drugs or interventions directed against each one of these four eco-evolutionary frames. A comprehensive list of these drugs and interventions is presented in Table 1.

Penetration inhibitors. (i) Inhibitors of high-risk bacterial clones. The local or global prevalence of AbR frequently depends on the local or global dissemination of particular bacterial clones which have been named high-risk antibiotic-resistant bacterial clones, eventually clustered in clonal complexes (152, 155). Note that the term "high-risk" is not necessarily associated here with clones causing severe infections. The "high-risk" clone emerges when a clone with high interhost transmission and colonization efficiency acquires an antibiotic resistance trait. Well-established examples of such clones (multilocus sequence clonal complexes [CCs] or sequence types [STs]) among Gram-positive bacteria are *Streptococcus pneumoniae* clonal complex 256 (CC256) and CC15 clones involved in the spread of beta-lactam and macrolide resistance, methicillin-resistant *Staphylococcus aureus* belonging to CC8 (sequence type 8 [ST8], ST239, and ST247), CC22 (ST22), CC5 (ST5 and ST228), CC45 (ST45), and CC30 (ST36); vancomycin- and/or ampicillin-resistant *Enterococcus faecium* belonging to the CC17 polyclonal subcluster (ST16, ST17, ST18 among others), or multiresistant *Enterococcus faecalis* clones corresponding to CC2 (ST2 and ST6), CC87 (ST87), and ST16 (152), some of which are shared by humans and farm animals (54). Examples among Gram-negative bacteria are *E. coli* clones such as O25b-B2-ST131 (113), *Klebsiella pneumoniae* CC258 (ST258 and ST11), some expanded clones of other enterobacterial species that are involved in the dissemination of resistance to extended-spectrum cephalosporins, or selected strains of multidrug-resistant (MDR) *Acinetobacter baumannii* (155). Changes in the overall prevalence of AbR in particular settings frequently occur because of the more or less efficient penetration of a relatively small diversity of such high-risk clones.

Direct fight against these clones or clonal complexes should reduce AbR. This has occurred with *Haemophilus influenzae* or *Streptococcus pneumoniae*. The introduction of the 7-valent conjugated vaccine (PC7) is in fact the first and most successful intervention in reducing AbR that has ever happened and should constitute a model for future eco-evo strategies. In fact, this success was due to the fact that PC7 reduces pharyngeal colonization of resistant serotypes in humans (infected or not) and blocks human-to-human spread of resistance (13). Targeted vaccination, or immunoserum protection of human populations at risk of being invaded by resistant clones, and then acting as sources for novel propagations, should be considered a current need in the prevention of AbR (31, 65, 145). Such types of immune protection could in the future include vaccination against colonization factors (for instance adhesins) of the resistant clones (38). Modeling methods to predict the effects of vaccination in different populations have recently been proposed (130). Certainly other fields of research are open for future development in therapy directed against resistant clones, including the use of resistant-clone-specific bacteriophages. Bacteriophages have been shown to work well in controlling bacterial dissemination when used in cocktails of different phages, avoiding the appearance of phage resistance (79, 98). Other possibilities are bacteria producing toxins that kill members of the same genotype or other bacterial (probiotic-like) organisms with clone-interfering abilities, serving as "Trojan horses" that interfere with the social (interactive) evolution of microorganisms (21, 27; Alex Mira, personal communication).

Ecological and evolutionary links exist between AbR and bacterial virulence (87). Virulent clones causing infections are frequently highly transmissible, have increased risk of being exposed to antibiotics, and consequently have the propensity of acquiring AbR (53a, 99). Consequently, targeting virulence might result in a decrease of antibiotic resistance. Even if they are out of the scope of this minireview, there is a wealth of new approaches to reduce bacterial virulence (reviewed in references 11, 12, and 26). Examples are methods targeting type III secretion inhibitors (1, 11, 12, 67) or quorum-sensing mechanisms (74, 78, 105, 111), and drugs able to modify bacterial master regulators influencing the expression of colonization factors of resistant clones can be expected (77, 118). Recently, screening chemicals against drug target enzymes predicted using a genome-scale metabolic network model has been proposed (118). This approach would presumptively allow analysis of the genome of any microbial pathogen and the development of a systems biological strategy by using genome-scale metabolic modeling and simulation for effective specific drug targeting and discovery (70).

(ii) Reduction of selective environments for high-risk resistant clones. Penetration of high-risk resistant clones is favored by antibiotic agents, as they reduce the "colonization resistance" of the recipient microbiota, in the classical van der Waaij approach (132, 144). Of course, much more needs to be known about the biological bases of suppression of invading populations by complexes of resident populations, a problem of systems biology (21). Metagenomics of human gut symbionts reveals a set of genes critical for adaptation to the gut environment and mechanisms for horizontal transfer of the genes (160). In any case, the reduction in the local antibiotic effect on indigenous microbes should be minimized. Novel molecules enhancing the upper-gut absorption of oral antibiotics or decreasing its biliar excretion should be considered to act against AbR. The strategy of destroying antibiotics in the intestine by the uptake of specific detoxifying enzymes, as beta-lactamases in patients treated with beta-lactam agents, or antibiotic-binding substances has been proposed (http://www.davolterra.com/rd-pipeline).

Promiscuity inhibitors. (i) Inhibitors of interbacterial dissemination of genetic elements. Genes encoding AbR frequently spread by means of MGEs. These elements are usually disseminated by any of the three classical bacterial DNA transfer mechanisms: conjugation, transformation, or transduction. Second-order transfer mechanisms also contribute to this spread, as plasmid "conduction," that is, cointegrate formation of a nonmobile element with a transmissible one and then resolution. Dissemination frequently occurs among "genetic exchange communities," which would be better named "genetic interactive communities" as there might be

TABLE 1. Possible eco-evo inhibitors of MDR bacteria and modes of action and effects

General target	Intervention	Specific target	Example(s) of inhibitors	Mode(s) of action	Current state of the art[a]	Implementation[b]	Reference(s)
Penetration	Inhibition of high-risk clonal complexes	Colonization factors	Synthetic multivalent glycoconjugates (lectin inhibitors)	Binding with specific lectins	2	2	38
		Surface antigens	Oral-mucosal vaccines. *Streptococcus pneumoniae* PCV7 and PCV13	Induction of protective mucosal cellular topical immunization strategies	3	2 or 3	31, 65
		Quorum sensing (QS)	Anthranilic acid analogues, acylated homoserine lactones (AHLs), marine water-derived QS inhibitors (fimbrolides, manoalides, sesterterpenoids), analogs of acyl-homoserine lactones (AHLs),[c] tetramic and tetronic acids, halogenated furanones	Prevent the expression of QS-regulated virulence/colonization genes. Removal of AHL; AHL antagonists; competitive binding of AHL receptors	2 and 3	2	14, 66, 78, 105, 111, 131
		Regulators of colonization factors	Rho GTPase-activating bacterial toxins?	Unknown	1	2	11, 77
		Variable targets	Drug targets predicted by systems biological strategy by using genome-scale metabolic modeling and simulation for effective drug targeting	To be established depending of the target	1	2	70, 118
	Reduction of selective environments	Antibiotics in upper gastrointestinal (GI) tract	Beta-lactam agents, or antibiotic-binding substances	Destroying antibiotics in the intestine by the uptake of specific detoxifying enzymes	3	2 (phase I in 2011)	www.davolterra.com/rd-pipeline
Promiscuity	Broad-host-range conjugation inhibitors (COINS)	Type IV secretion systems?	Unsaturated fatty acids (e.g., dehydrocrepenynic acid, linoleic and linolenic acids)	Unknown	2	1	50
		Type IV secretion systems (VirB11-type nucleotide triphosphatases [NTPases])	Thiadiazolidine-3,5-diones (compounds belonging to the Chiron Corporation substance libraries)	Inhibition of VirB11-type NTPases	2	2	64
		Type IV secretion systems (relaxases)	Specific biphosphonates clodronate (Bonefos) and etidronate (Didronel)	Inhibition of relaxases. Killing F^+ cells and preventing conjugative DNA transfer	2	1	84
		Type IV secretion systems (VirB8-type)	Salicylidene acylhydrazide (structurally similar to a class of molecules that have broad-spectrum activity against type III secretion [T3S]) systems	Inhibition of VirB8 interactions with other type IV secretion (T4S) components via its periplasmic C-terminal domain by targeting dimerization	1	1	105
	Specific conjugation inhibitors	Type IVB pili	Aptamer single-stranded DNA (ssDNA) and single-stranded RNA (ssRNA)	Binding to type IVB pili and further inhibition of the entry of the piliated strain (but not that of the nonpiliated strain) into human THP-1 cells	1	1	104
		Type IV pili	Bacteriophages	Unknown	1	1	98
		Entry exclusion proteins, pheromones	Vaccine, aptamers	Unknown	1	1	57
		Restriction systems	Vaccine, aptamers	Unknown	1	1	141
		CRISPRs (regularly interspaced short palindromic repeats)	Vaccine, aptamers	Unknown	1	1	86
		CRISPRs (regularly interspaced short palindromic repeats)	Vaccine, aptamers	Unknown	1	1	86

Plasticity	Recombinase inhibitors	RecA	2-Amino-4,6-diarylpyridine compounds	Inhibition of RecA-mediated strand exchange and bacterial SOS response in live bacterial cell cultures	1	1	117, 151
		Integrase	Putative integrase inhibitors (as in HIV)		1	1	58
	Mutation inhibitors	LexA (SOS gene networks)	Bacteriophages expressing uncleavable LexA variants (e.g., engineered M13 phage with *lexA3* repressor)		1	1	83
		SoxRS (non-SOS-gene networks)	Bacteriophages overexpressing SoxRS	Overexpression of proteins to target non-SOS gene networks. Prevention of mutagenic events by superoxides	1	1	83
Persistence	Decontamination of high-risk clones/clonal complexes	Anticlonal vaccines	*Streptococcus pneumoniae* PCV7 and PCV13	Antibody-mediated inhibition of mucosal colonization	3	2/3	65
		Essential genes for growth or virulence (e.g., *acpP*, an acyl carrier protein of *Escherichia coli* or *Burkholderia cepacia*)	Antisense oligomers peptide-conjugated PMOs (PPMOs), cationic PMOs (Gux-PMOs, Pip-PMOs) (PMOs are phosphorodiamidate morpholino oligonucleotides)[c]	Targeting *acpP* (acyl carrier protein) essential for growth	2	2	61, 88
		Clonal interference strategies	Genetically modified organisms (GMOs), probiotics	Biological containment systems based in different interference strategies (use of colicins or other amensalistic substances, competition in a common niche)	2/3	2	94, 142
	Decontamination of mobile genetic elements (MGEs)	Plasmid decontamination	Phenothiazines (such as promethiazine), dibenzoazepines, dibenzocykloheptene, plumbagin	Not defined theoretically, based on dfferential DNA topology in plasmids or the bacterial chromosome, and/or the membrane-plasmid DNA interaction during replication	1	1	90, 102, 128, 129
		Exploiting plasmid incompatibility mechanisms (replication machinery)	Different inhibitors and approaches validated only in the laboratory	Not defined	1	1	37, 95, 139, 147
		Exploiting toxin-antitoxin (TA) systems	Homologs of the *hok* plasmid mantenance factor. Biological containment systems. GMOs carrying TA homologs	Homologs of RelF for the control of *Escherichia coli.* Homologs of GeF for the control of *Pseudomonas putida.*	2	2	71, 89, 114
	Decontamination of high-risk genes	Antibiotic resistance genes	Antisense oligomers, such as phosphorothioate oligonucleotides (PS-ODNs), locked nucleic acids (LNAs), 2′-*O*-methyloligoribonucleotides (2′*O*Mes), phosphorodiamidate morpholino oligonucleotides (PMOs), and peptide nucleic acids (PNAs)	Targeting gene antisense and antigene oligonucleotides bind mRNA to prevent translation or bind DNA to prevent gene transcription. Interruption of the expression of resistance genes to restore susceptibility to key antibiotics	2	2	125, 156
		Antibiotic resistance genes, metabolic genes	Short double-stranded RNAs (small interfering RNA [siRNA])	siRNAs are incorporated into an RNA-induced silencing complex that directs degradation of RNA containing a homologous sequence	2	2	23, 120

[a] The current state of the art is shown as follows: 1, theoretical approach; 2, *in vitro* evidence; 3, *in vivo* evidence.
[b] The possibility of implentation is shown as follows: 1, difficult; 2, possible; 3, accessible.
[c] Pip, piperazine; Gux, *N*-(6-guanidinohexanoyl)piperazine.

unidirectional transfer (121). Such ensembles of bacteria frequently (but not always) have ecological compatibility and genomic similarity, which facilitates genetic transfer, homologous/homeologous recombination, expression, and subsequent regulation (41, 121). The application of interference strategies to genetic exchange networks, and particularly interference of "broker clones" able to spread plasmids very efficiently in heterogeneous bacterial communities (42), eventually by the use of probiotics (93) should reduce genetic promiscuity. The important point here is that the natural history of AbR indicates that conjugation has been the predominant mechanism used by resistance genes to appear in many human pathogens. Once there, the genes can be dispersed further by transformation and/or transduction. The main reason for the predominance of conjugation might be that this process is naturally broad host range, certainly much broader than transformation and transduction (122).

(ii) Broad-host-range conjugation inhibitors. Conjugation effects the dissemination of plasmids, conjugative transposons, and ICEs. Moreover, most plasmids (and related elements) conjugate using variants of the same biochemical core process. Specifically, they all use a protein called relaxase to bring about the conjugative replication of the DNA to be transferred and a set of ATPases to motor the process of DNA transport. Therefore, although other targets will be discussed below, conjugation seems to be the preferred potential target, thanks to its universality (36, 122).

The fact that there is a single predominant mechanism of plasmid conjugation raises the hope that broad-host-range anticonjugation drugs (called COINS hereafter, for conjugation inhibitors) can be discovered. There have in fact been important advances in this direction. First, a high-throughput whole-cell assay to detect and assay for COINS was developed (50). Using this assay, a conjugation-inhibitory compound called dehydrocrepenynic acid, an unsaturated fatty acid, was discovered, establishing the proof of principle that this type of eco-evo drugs can be found. It was also found that other polyunsaturated fatty acids, like linoleic and linolenic acids, are also potent COINS. Later on and using screening methods for *in vitro* reactions, inhibitors were found for both relaxase proteins (84) and one of the conjugative motor ATPases (64). Antirelaxase compounds (biphosphonates, clodronate, and etidronate) worked efficiently *in vitro*, were much less effective *in vivo*, and presented unexpected results on cell viability (84). Another line of research is the investigation of components of the *E. coli* cell wall that inhibit conjugation of the classical F-factor. These components were solubilized and partially purified. Up to now, their nature and potential applications are unknown (116). Finally, there is a recently described mechanism for the control of dissemination of plasmids and phages, which is effected by regularly interspaced short palindromic repeats (CRISPRs) (86, 127) These DNA elements, when located in the recipient bacteria, interfere with conjugation and plasmid transformation, for instance in *Staphylococcus* (86) The range of their activities is necessarily narrow, as they use specific sequences that have to match perfectly to the target to activate the nucleases.

(iii) Specific conjugation inhibitors. As in the case of "high-risk clones" (discussed above), we can also consider "high-risk plasmids," plasmids that efficiently disseminate among bacterial organisms (particularly among "genetic interactive communities") and harbor dangerous resistance genes (22, 121, 122, 135). Advances in identification of such target MGEs might provide alternatives directed to specific conjugation systems. (i) Some bacteriophages specifically bind different types of pili involved in bacterial conjugation (98). In all cases, the phages target specific pilus types, and thus, their range of targets is limited. However, using the same principle as in antibacterial phage treatment, enlargement of the spectrum of inhibition could be expected for a cocktail of phages, each with specificity for a major potential target, (79). Similar antipili strategies could be devised with the use of aptamers (69, 104). (ii) Another possibility is based on entry exclusion proteins, a diverse family of small proteins known to be efficient inhibitors of conjugation, which could be considered for future anticonjugation vaccines or aptamers developed by synthetic biology (57). (iii) Techniques that exploit the phenomenon of DNA restriction could be used. Restriction systems can be added to inhibit plasmids (perhaps in combination with the *eex* genes) to broaden and potentiate the mechanisms of inhibition (84, 141). (iv) Methods that inhibit modulators of bacterial conjugation could be used. Some bacteria, like *Enterococcus faecalis*, produce pheromones that are necessary to induce plasmid conjugation. Antipheromone drugs or vaccines could limit the expansion of particular plasmids. Of course, similar strategies could be devised to decrease bacterial transformation, particularly targeting competence proteins and/or recognition sequences in transformed DNA. (v) Some plasmids, like plasmid F, have inducible transfer systems that are usually repressed (fertility inhibition systems). Inhibitors of the mechanisms of induction of fertility should obviously decrease plasmid promiscuity.

Plasticity inhibitors. The evolution of AbR depends on the mechanisms involved in genetic variability, as mutation and recombination. Frequent recombination-modularization events (4, 41) contribute to the building up of complex genetic structures in which resistance genes are located. Such platforms often include other resistance genes, thus increasing the ability of the genetic ensemble to be selected by various antimicrobial agents.

(i) Recombinase inhibitors. Inhibition of recombination is a hypothetical target that should decrease the acquisition and integration of new resistance genes and platforms, delaying the evolution of the complex genetic structures involved in multiresistance. RecA protein plays important roles in a number of DNA recombination and repair processes, including homologous recombination and recombinational DNA repair. Small molecule RecA inhibitors may sensitize bacteria to established antibacterial agents and also prevent the development and acquisition of genes conferring drug resistance. For instance, tandem-gene amplification, a key first step for evolving resistance, is RecA dependent (Dan Andersson, personal communication). In *Bacillus subtilis*, DNA uptake machinery requires RecA for integration of homologous donor DNA into the recipient chromosome, while plasmid establishment, which is independent of RecA, requires at least RecO and RecU (68). The search for site-specific recombination inhibitors (focused on transposon Tn*3*) started a quarter of century ago (49). Possible metal-chelate RecA inhibitors have been proposed as transition metal complexes (based on metal-dependent initia-

tion of RecA aggregation), nucleotide analogs, structured peptides, and polysulfated naphthyl compounds (75, 76, 151). Unfortunately, these molecules are not cell permeable or have a pleiotropic effect on live bacteria. Recently, a high-throughput target-based screening approach has permitted the identification of a new class of small, permeable compounds that can attenuate the SOS response in living bacteria (117, 150, 151), Conjugative plasmids have a natural RecA inhibitor, PsiB (108), and research in natural systems of controlling recombination will eventually be of interest. Finally, investigation on HIV-1 has yielded a large number of integrase inhibitors, which eventually could be tested for inhibition of resistance gene acquisition by genetic platforms (58; J. C. Galán, personal communication).

(ii) Mutation inhibitors. The possibility of controlling mutations due to either DNA damage repair or reactive oxygen species (ROS)-forming systems as a novel therapeutic strategy has already been proposed (25, 46, 72, 138). Most RecA inhibitors discussed in the above paragraph should have an inhibitory effect on SOS induction, therefore reducing the mutational capabilities of the bacterial cell. Cleavage of the SOS repressor LexA is induced by antibiotic DNA damage resulting from superoxides resulting from the metabolic changes induced by antibiotic action (46, 73). LexA cleavage produces the derepression of RecA and the SOS-regulated polymerases Pol II, Pol IV, and Pol V, promoting recombinational and mutational changes leading to AbR. It has been suggested that inhibition of these proteins or prevention of their derepression by inhibition of LexA cleavage with suitably designed drugs might represent a fundamentally new approach to combating the emerging threat of AbR bacteria (24). The reduction in the intracellular concentrations of mutagenic compounds, as DNA-damaging superoxides, should also reduce mutation rates. The proof of concept is provided by the observation that the overexpression of a *norM* multidrug and toxin extrusion (MATE) family efflux pump in bacteria protects against reactive oxygen species and provides antimutability (62). Combination therapy coupling antibiotics with antibiotic-enhancing bacteriophage engineered to target SOS (by expressing uncleavable LexA variants) or non-SOS gene networks (by overexpressing SoxR of the superoxide-responsive system SoxSR) significantly increases survival in antibiotic-treated animals and has been suggested as a promising antimicrobial approach (46, 83). Drugs that might increase bacterial superoxide dismutases could have similar effects. Extracellular stress-induced mutation (and eventually resistance gene amplification) also depends on RpoS, sigma 32, and sigma E (59). The possibilities of upregulation of the methyl-mismatch repair (MMR) systems as a way of decreasing mutational adaptation are poor, as even in multicopy, the genes of the MMR system scarcely influence mutation rates (56).

Persistence inhibitors. (i) Decontamination of high-risk clones. Eco-evo drugs and strategies directed to reducing the absolute number of high-risk clone cells could have important consequences in fighting AbR. A high-risk clone is a multidrug-resistant clone with highly efficient transmission and/or maintenance among humans or animals. Even if it is beyond the scope of this minireview, it is obvious that hospital hygiene, antibiotic, and disinfectant policies and containment activities contribute to the hospital decontamination of high-risk resistant clones, but as stated before, such an approach has had limited success, particularly in heavily contaminated areas (17). A number of antipenetration eco-evo drugs might reduce persistence, as clone-directed vaccines, antisense oligomers targeting clone-specific genes (discussed below, antigene approaches), specific immunosera, phages, or substances decreasing the selective activity of antibiotics, for instance on the intestinal microbiota (see above). A number of bacterial traits involved in persistence are frequently considered "virulence traits," so that advances in some of the new molecules targeting virulence might also influence bacterial persistence of high-risk clones (12, 26). Another interesting possibility will be to use clonal interference strategies. *E. coli* probiotic strains producing mixtures of microcins (small antibiotic peptides), such as microcins M and H (142) could be good potential candidates for this task. Genetic modified organisms (GMOs) carrying amensalistic substances have been already used in experimental remediation with *Pseudomonas putida* (94).

(ii) Decontamination of high-risk MGEs. The decontamination of high-risk MGEs is undoubtedly a difficult objective. Reduction in the absolute number of high-risk clones harboring MGEs does not necessarily ensure a significant reduction of high-risk mobile elements. Such MGEs might be harbored within multiple species and clones forming genetic exchange communities (121), thus acting as potential reservoirs for the maintenance of MGEs. An important recent approach is the possible use of bacterial probiotic clones that interfere with plasmid exchange networks but that are unable to be invaded by high-risk MGEs (21, 107). A more straightforward approach could be a direct attack on MGEs, like plasmids, that could become "the Achilles heel of drug-resistant bacteria" (153), reconverting resistant bacteria into susceptible ones. The history of "plasmid curing approaches" began more than 30 years ago and is based on the observation of plasmid elimination by acridine dyes (recently confirmed with 9-aminoacridine), ethidium bromide, or sodium dodecyl sulfate (129).

More recently, two main approaches have been applied for "plasmid decontamination" of bacterial populations. The earliest (but still active) line of research has investigated antiplasmid compounds that take advantage of the differential DNA topology in plasmids or the bacterial chromosome and/or the membrane-plasmid DNA interaction during replication. Plasmid DNA exists in a superhelical state more frequently than chromosomal DNA. For instance, heterocyclic compounds bind better to supercoiled plasmids, differentially inhibiting their replication (129). Tricyclic molecules, such as phenothiazines (as promethiazine), dibenzoazepines, dibenzocycloheptene derivatives, and some stereoisomers, have been able to cure *E. coli* plasmids *in vitro* (90). The relatively low efficiency in plasmid elimination might be increased by the association with calmodulin or proton pump inhibitors (128). Plumbagin, a simple plant secondary metabolite, seems to eliminate stringent, conjugative, multidrug-resistant plasmids from several strains (102).

The second and more recent approach exploits mechanisms involved in natural plasmid elimination (2) and is discussed below in more detail. Natural plasmid loss occurs essentially by the following mechanisms: (i) excessive biological cost for the host cell in maintaining the MGE, so that spontaneously plasmid-free cells overcome plasmid-bearing cells; (ii) plasmid in-

compatibility, when plasmids of the same incompatibility group (depending on their *rep* sequences, the replication machinery) do not stably cosegregate to a daughter cell; (iii) plasmid failures in partition and multimer resolution, eventually giving rise to plasmid-free daughter cells; (iv) when bacteria lose their protection against toxins produced by plasmids themselves (toxin-antitoxin systems). Indeed, each one of these mechanisms might provide clues for the development of future MGE decontamination drugs.

Plasmid cost enhancers. The reasons behind plasmid biological cost remain poorly investigated. It is known that such a cost frequently peaks after the acquisition of a novel plasmid for the host organism, and the cost is progressively reduced during coexistence time, in a kind of "plasmid domestication" (35). Domestication probably results from a complex rewiring of the cell metabolism involving the presence of the plasmid. The cost frequently increases upon expression of the resistance gene (for instance, after transcriptional induction), and this cost is variable for different genes (96). This suggests that there could be a window of opportunity for cost enhancer drugs when we try to suppress the spread of a plasmid carrying particular genes from a successful penetrating strain to other strains of different clones or species. A more sophisticated approach could be the artificial introduction of a selectable gene (for instance a gene encoding a nonabsorbable, nontoxic, but nontherapeutical antimicrobial agent or a gene encoding a natural amensalistic substance, bacteriocin, or microcin) in a bacterial community by means of particularly efficient plasmid vehicles or biodrugs, using the acquisition of that gene in the presence of the selector to increase the overall cost of the recipient plasmids.

Exploiting plasmid incompatibility mechanisms. The exploitation of the mechanisms of plasmid incompatibility has been explored (37, 139, 153). In this case, the plasmid replication machinery is an obvious target, for instance using compounds able to reduce the intracellular levels of RepA replicase. These levels are determined by its interaction with a small RNA, RNAI, whose conformation can be altered by drugs, such as apramycin, at least in incompatibility IncB systems (139). An apparent limitation to such an approach is the possible rapid emergence of plasmid elimination-resistant mutants by mutational changes in the Rep sequences. As in the previous case, we can imagine the artificial introduction and selection within a particular bacterial community of a plasmid encoding a nontherapeutic antimicrobial resistance trait, which could eventually be able to displace plasmids of the same incompatibility group. The introduction and maintenance of the resistance-curing plasmid-selecting agent would probably require the administration of a nontherapeutic compound selecting for the new plasmid.

Exploiting toxin-antitoxin systems. A number of clinically important plasmids containing antibiotic resistance genes have toxin-antitoxin (TA) systems. The plasmid simultaneously produces a stable toxin able to kill the bacterial host and a labile antitoxin, so that if the plasmid is lost, the antitoxin is not longer produced, and the toxin that remains in the bacterial cytoplasm induces cell death. This kind of host capital penalization for plasmid loss, which has also been called "mafia strategy" (96), forces the coexistence of the plasmid and the cell harboring the plasmid, facilitating metabolic networking between both replicons, and finally leading to plasmid domestication. Compounds that can increase the production of the toxin, decrease the production of the antitoxin, or interfere in the toxin-antitoxin interaction will result in the loss of cells containing the plasmid (40, 81). A possible limitation of this strategy is the presence of toxin-antitoxin systems in the bacterial chromosomes. For instance, induction of chromosomal TA genes results in an increase of bacterial cells presenting a "persistence" phenotype, with cells that neither grow nor die in the presence of antimicrobial agents but that are able to reassume division once the exposure is over (43, 80). Obviously, in the absence of death or replication, plasmid loss would be severely limited.

Finally, partition and multimer resolution could theoretically be considered processes with potential antiplasmid targets, but as far as we know, nothing has been proposed or published on this possibility. It is of note that many of the possible antiplasmid resistance drugs will have a very limited target, a plasmid or a plasmid family. The possible utility of such products will be specific plasmid decontamination, a goal that fits well with targeted eco-evo therapy.

Decontamination of high-risk genes. The possibility of decontamination of a bacterial system of particular AbR genes or eventually critical genes for the maintenance or spread of MGEs carrying resistance determinants has also been examined. The use of antisense oligomers (ASOs) is a promising technology for treating viral and bacterial infections (148). A major class of ASO compounds, phosphorodiamidate morpholino oligonucleotides (PMOs), target bacterial genes known to be essential for growth or virulence have recently been proved efficient in treating chronic infections caused by multidrug-resistant *Burkholderia* species complex (26, 61). A similar strategy could be applied to inactivate antibiotic resistance genes (156). Antisense strategies directed toward specific resistance genes (such as *aac(6′)-Ib*) have been successfully applied to reduce AbR (amikacin resistance) (125). Recently, small interfering RNAs have also been considered "therapeutic targets" for gene silencing (23, 120), a concept of potential application to AbR. Note that inhibiting resistance at the nucleic acid level has been hitherto considered only to prevent resistance gene expression, the resistance phenotype, during therapy. Decontamination of resistance genes will require directed gene knockout by mutagenesis or gene disruption or allelic replacement by susceptible genes, which is a feasible *in vitro* approach, but it is difficult to foresee the methods to achieve effective gene replacement in the field and to select for the resulting susceptible variants.

Selection for antibiotic-susceptible bacterial organisms. Indeed, many of the strategies and possible eco-evo therapeutic approaches discussed above should produce a selection of antibiotic-susceptible organisms. However, other possibilities for very active selection should be considered. Let us imagine an antibiotic X that is active only upon a chemical modification that is available by the action of an enzyme that inactivates another antimicrobial agent Y, whose clinical use is aimed to be preserved. Only Y-resistant bacteria will be killed by the antibiotic X. The expected effect is selection for susceptibility. In a more classical way of thinking, the introduction of new antibiotics in hospitals might have a beneficial effect in the levels of resistance to previously used antibiotics, of course

under the condition of non-cross-resistance. In some cases, the evolution toward resistance to a given antibiotic increases susceptibility to another one, and vice versa, an antagonistic pleiotropy phenomenon (15, 97). Finally, we cannot exclude the possibility that a number of refined bacterium-decontaminating procedures (heat, radiation, novel biocides, or others) could be tuned to target preferentially antibiotic-resistant organisms. At this time, this seems a difficult task, but it must be mentioned.

Eco-evo or eco-evo-devo? Microbiosphere restoration. The eco-devo (evolutionary developmental biology) approach explores the mechanistic relationships between the processes of individual development (such as embryogenesis) and phenotypic change (such as morphotypes) during evolution (133). If a real "development" is rare in the individual bacterial cells, a kind of development occurs in complex microbial systems associated with humans, such as the gut microbiota, which can be considered a collective individual at a supraspecies level of the biological hierarchy, evolving (changing) with the host from birth to senescence. The eco-evo-devo (ecological evolutionary developmental biology) approach (136) perspective integrates development biology with ecology and evolution. Indeed, if we could influence the structure and development of the microbiota associated with humans, it should have an effect on the networking of genetic interacting communities sharing, mobilizing, and evolving resistance genes and platforms, and finally on AbR. Little has been done to correlate microbiotic structure with AbR, but there is certainly something important to delve into. We can consider the possibility of "microbiotic transplantation" with susceptible organisms early in life or after appropriate decontamination procedures. For such a purpose, we need to know more about the microbiota structure and its relations with the host genetics and environment. However, considering the speed of evolution of AbR, the time might have arrived to start thinking about biobanks able to preserve susceptible microbiotic ensembles for future biorestorative interventions.

Eco-evo interventions: indications, ethics, models, and outcomes. The main set of problems for the development of eco-evo interventions is related to the need for a change in society about the concept of "what is a drug." From drugs that cure patients to drugs that cure a sick environment, there is certainly a big jump both for the industry and regulatory agencies. As in the case of sick patients, sick environments should be diagnosed before treatment. We have here an important challenge—what is a sick environment, in our case, what is a sick environment because of antibiotic resistance? In other words, could we establish "indications" for eco-evo local interventions? A typical case could be a intensive care facility (or a farm) where a particular clone of a pathogenic organism has been isolated with a novel, potentially dangerous mechanism of resistance encoded by a gene hosted by a particular integron located in a particular transposon, hosted by a highly transmissible plasmid. We are also aware (thanks to multilevel epidemiology tools) that the plasmid has been transferred to commensal microorganisms and the clone has been transferred from the index patient to other patients. Another possibility are more-restricted sick environments, such as the bronchial spaces in cystic fibrosis patients, where prevention of hypermutation or recombination and/or anticlonal vaccination could be used. These are sick environments for antibiotic resistance.

Current clinical trials with new drugs under development are oriented almost exclusively to measure their effects on individuals (human volunteers or sick patients). Eco-evo interventions are mostly (but not exclusively) oriented to modify the "risk landscape," in our case for AbR. We have reviewed here and in Table 1 a number of possibilities for controlling the ecology and evolution of AbR inside and outside the human host. Indeed, eco-evo interventions to reduce AbR in the environment should be carefully controlled using environmental risk assessment standards but refined to be able to detect small-scale but eventually important changes. Assessment of eco-evo interventions will require the development application of novel technologies for investigating the appropriate outcomes. The quantification of the effect of eco-evo interventions in changes in the frequency of AbR in individual patients (such as strongly immunosuppressed patients), small areas (such as intensive care units), larger organizations (such as hospitals, long-term care facilities, and farms), or the community will also require appropriate tools. An important issue is the possible adverse events of eco-evo interventions trying to limit AbR. A number of the possible compounds discussed in this minireview, particularly those aiming to control the molecular mechanisms involved in mutational or recombinational events, will probably have at this time low selectivity, as human cells have similar targets. Undesirable and even unexpected effects on natural microbial ecosystems should be also considered. For instance, populational replacements could be provided (as with the PC7 vaccine in *S. pneumoniae* populations). If nonspecific plasmid-targeting drugs are used, their impact on the productivity and biodegradation capability of environmental microbiota should be considered. Indeed, there is a possibility of using collective animal models (experimental farms and their environment) to test eco-evo interventions and using the data obtained to feed appropriate mathematical models. In this respect, it is of note that many of the possible eco-evo interventions could be conceived as very targeted (narrow spectrum), focusing for instance on particular genetic exchange communities, clonal complexes, strains, plasmids, or genes. Such a "biosurgical" approach should also be permeated into the conservative minds of the pharmaceutical industry and regulatory agencies.

Concluding remarks. It is our hope that this minireview will help the scientific and industrial communities to realize that the eco-evo perspective offers a number of potential outcomes that can be used to slow down the evolution and dissemination of AbR and eventually to partially restore susceptibility in particular environments. With a few exceptions (anticlone vaccination), at this time we have only a small number of promising products and almost no experience in the possible effects of the eco-evo strategy in the real world, but interest has started to rise in the pharmaceutical and ecological industry. In the years to come, relevant laboratory and field experiments should be carried out to prove that any of these approaches can be effective in reducing the frequency or limiting the dissemination of the genes and genetic platforms involved in AbR. However, our work will be inconsequential if it does not contribute to permeate the thinking of scientists and regulatory agencies with the idea that there is a need to consider a space

for a new type of "drugs" as agents of future eco-evo interventions to decrease AbR. Finally, it should be clear once more that AbR is a "model problem," and similar eco-evo strategies could be probably applied in the future for maintenance of a healthy interaction between humans and microbial systems in a rapidly changing biosphere.

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