Indirect resistance to several classes of antibiotics in cocultures with resistant bacteria expressing antibiotic-modifying or -degrading enzymes

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Objectives: Indirect resistance (IR), the ability of an antibiotic-resistant population of bacteria to protect a susceptible population, has been previously observed for β-lactamase-producing bacteria and associated with antimicrobial treatment failures. Here, we determined whether other resistance determinants could cause IR in the presence of five other classes of antibiotics.

Methods: A test was designed to detect IR and 14 antibiotic resistance genes were tested in the presence of 13 antibiotics from six classes. A bioassay was used to measure the ability of resistance-causing enzymes to decrease the concentration of active antibiotics in the medium.

Results: We confirmed IR in the presence of β-lactam antibiotics (ampicillin and mecillinam) when TEM-1A was expressed. We found that bacteria expressing antibiotic-modifying or -degrading enzymes Ere(A), Tet(X2) or CatA1 caused IR in the presence of macrolides (erythromycin and clarithromycin), tetracyclines (tetracycline and tigecycline) and chloramphenicol, respectively. IR was not observed with resistance determinants that did not modify or destroy antibiotics or with enzymes modifying aminoglycosides or degrading fosfomycin. IR was dependent on the resistance enzymes decreasing the concentration of active antibiotics in the medium, hence allowing nearby susceptible bacteria to resume growth once the antibiotic concentration fell below their MIC.

Conclusions: IR was not limited to β-lactamase-producing bacteria, but was also caused by resistant bacteria carrying cytoplasmic antibiotic-modifying or -degrading enzymes that catalyse energy-consuming reactions requiring complex cellular cofactors. Our results suggest that IR is common and further emphasizes that coinfecting agents and the human microflora can have a negative impact during antimicrobial therapy.

Introduction

Indirect resistance (IR), also known as indirect pathogenicity or passive resistance, is the ability of a population of antibiotic-resistant bacteria to protect a population of susceptible bacteria during coinfection. Clinically, IR involves an antibiotic-resistant population protecting a pathogenic antibiotic-susceptible population that is the intended target of the antimicrobial treatment. IR was first described by Maddocks and May1 in 1969 when they observed that a penicillin-susceptible Haemophilus influenzae strain was protected in vivo and in vitro by β-lactamase-producing members of Enterobacteriaceae during mixed infections. The H. influenzae and Enterobacteriaceae coinfecting agents were then efficiently cured by use of penicillin and the β-lactamase inhibitor cloxacillin. Failure of treatments with β-lactams in cases of coinfections with β-lactamase-producing bacteria had already been reported in earlier work.3 Since then, IR has been implicated in numerous antimicrobial treatment failures in which a β-lactam-resistant population protected the pathogen.3–5 To date, all described cases of IR involve organisms producing β-lactamases, which are enzymes catalysing the hydrolysis of β-lactam antibiotics. Several characteristics differentiate β-lactamases from other antibiotic-modifying or -degrading enzymes: (i) the hydrolysis mechanism does not consume energy and involves water molecules instead of complex cell-synthesized cofactors; and (ii) the enzyme is located in the periplasm or excreted into the medium where it remains active rather than sequestered in the cytoplasm.6–8 Both the periplasmic and released enzymes contribute to decreasing the concentration of active antibiotics in the medium, which allows surviving antibiotic-susceptible cells located near the β-lactamase producers to resume growth once the concentration of drug falls below the MIC for the susceptible population. It was hypothesized that IR might be specific to β-lactamase-producing bacteria because of the specificities of those enzymes and to date no IR was described...
that involves resistance enzymes modifying or degrading antibiotics of other classes. Previous studies showed absence of IR to kanamycin and spectinomycin despite resistant populations carrying antibiotic-modifying enzymes.\textsuperscript{4,9} It was postulated that absence of IR might be due to the cytoplasmic localization of those antibiotic-modifying enzymes and/or to the high energy cost of the modification mechanism they catalyse.

In addition to production of $\beta$-lactamases, several other antibiotic-modifying or -degrading enzymes are responsible for the high resistance of environmental and clinical strains to antibiotics from several classes.\textsuperscript{10–12} Unlike $\beta$-lactamases, these enzymes are localized in the cytoplasm and the antibiotic modification/degradation reactions they catalyse are usually energy-consuming and involve complex cofactors. IR is a generally poorly understood phenomenon and, to our knowledge, it remains unclear if non-$\beta$-lactamase enzymes could cause IR. Investigating IR towards other classes of antibiotics is of great clinical importance as a broader prevalence of IR than currently postulated would further emphasize the negative impact that drug-resistant coinfecting agents or bacteria from the normal microflora might exert during antimicrobial therapy. Here, we studied IR when susceptible bacteria were cocultured with resistant bacteria producing antibiotic-modifying or -degrading enzymes other than $\beta$-lactamases and the parameters that affect the development of IR. Fourteen antibiotic resistance genes causing resistance by modification or degradation of the drug ($n = 10$) or other antibiotic resistance mechanisms ($n = 4$) were tested in the presence of 13 different antibiotics from the following classes: macrolides ($n = 2$), aminoglycosides ($n = 5$), tetracyclines ($n = 2$), $\beta$-lactams ($n = 2$), fosfomycin ($n = 1$) and chloramphenicol ($n = 1$). We found several cases of IR and discovered that IR was dependent on the ability of drug-modifying or -degrading enzymes to decrease the amount of antibiotic present in the extracellular medium, rather than on the localization of the enzymes or the antibiotic modification/degradation mechanism catalysed.

**Materials and methods**

**Media, antibiotics, strains and plasmids**

All growths were performed at 37°C. High salt LB broth and LB agar (LA) media were purchased from Fluka. M9 broth and M9 agar minimal media were supplemented with glucose and lactose (as below) and 2 mg/L thymine. Erythromycin, fosfomycin, gentamicin, tobramycin, kanamycin, streptomycin, spectinomycin, tetracycline, ampicillin and chloramphenicol were purchased from Sigma–Aldrich. Antibiotic stock solutions were prepared fresh before each experiment.

The strains used in this study are listed in Table 1. Genus of DA34369 (Micrococcus sp.) was determined by 16S sequencing (Table S1, available elsewhere).

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Strain</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micrococcus</td>
<td>sp.</td>
<td>DA34369</td>
<td>16S sequencing</td>
</tr>
</tbody>
</table>

All other strains and mutants are derivatives of Escherichia coli MG1655. accA was deleted by P1\textsubscript{wr} transduction of the $\Delta$acca::kan marker from the Keio collection clone JW0452\textsuperscript{13} into MG1655 (DA5438) and the kanamycin marker was cured using plasmid pCP20.\textsuperscript{14} The lacI2YA deletion was performed by $\lambda$ Red recombination using the kanamycin marker from plasmid pKD4\textsuperscript{14} and the PCR primers listed in Table S1. After P1\textsubscript{wr} transduction of the $\Delta$lacI2YA::kan construct into DA5438 and DA34574, the kanamycin marker was cured using plasmid pCP20.

The IPTG-inducible plasmid pCA24NCL2 (chloramphenicol resistance) is the ASKA collection plasmid pCA24N(gfp-)\textsuperscript{15} modified to remove the His-tag and introduce restriction sites compatible with the cloning of the resistance genes. All PCR amplifications for cloning purposes were performed with Phusion High-Fidelity DNA polymerase (Thermo Scientific) and primers were purchased from Eurofin Genomics. PCR products, digestions and ligation reactions were purified using the SureClean DNA purification kit (Bioline). pCA24NCL2 was constructed as follows: pCA24N(gfp-) PCR-amplified (Table S1) and the amplified product was digested with restriction enzymes XbaI, KpnI and DpnI (Fermentas). A double-strand DNA insert compatible with the XbaI + KpnI-digested plasmid was made by annealing oligonucleotides pCA24N_Ins_F and pCA24N_Ins_R (Table S1) and ligated into the digested plasmid. Ligation was transformed into E. coli DH5\textsubscript{a} and constructs were verified by sequencing. Plasmid pCA24NKM2 (kanamycin resistance) was constructed as follows. The npt gene was amplified from plasmid pKD4 (Table S1) and introduced into pCA24NCL2 by $\lambda$ Red recombination to replace the cat gene and obtain pCA24NKM2.\textsuperscript{16} The different resistance-encoding genes were PCR-amplified using primers and template DNA described in Table S1. Amplified products were digested with XbaI + KpnI or XbaI + SpH\textsubscript{i}, ligated into plasmid pCA24NCL2 digested with the same restriction enzymes (Table S1) and transformed into E. coli DH5\textsubscript{a}. Constructs were verified by sequencing before electroporation into strains DA26577 and/or DA39350. Plasmid pCA24N(gfp-)::mura was prepared from the ASKA collection clone JW3156\textsuperscript{17} and electroporated into DA26577. Plasmids pCA24N(gfp-)::bla\textsubscript{TEM-1A}(A) and pCA24N(gfp-)::bla\textsubscript{TEM-1A}(B) had been selected in the presence of tetracycline, ampicillin or mecinilam, respectively, during a metagenomics project in our laboratory and were electroporated into DA32739. Plasmids pPlIP1100\textsuperscript{18} and pAT63 were electroporated into DA32739 and/or DA39350 and selected on 100 mg/L ampicillin. Plasmid p147 was conjugated into DA32739 from donor clinical isolate 147\textsuperscript{17} and selected on 30 mg/L chloramphenicol.

**IR test**

We designed a test that allowed us to detect IR on agar plates. This test required growth conditions allowing us to differentiate two populations of bacteria mixed and grown together on agar plates. Under such conditions, the effect that one population of bacteria (typically the antibiotic-resistant population) might have on the MIC for the second population of bacteria (the antibiotic-susceptible population) can be monitored. Two different sets of strains and media were used. The first set involved cocultures of antibiotic-resistant Gram-negative E. coli mixed with antibiotic-susceptible Gram-positive Micrococcus sp. and the MIC for each population was determined on LB agar plates. The Micrococcus strain used in this study was originally isolated as a contaminant and in subsequent studies used because of: (i) its ability to give colonies easily distinguishable on a lawn of E. coli MG1655; and (ii) its susceptibility to several antibiotics. The second set involved cocultures of antibiotic-resistant E. coli lactose\textsuperscript{−} (Lac\textsuperscript{−}) mixed with antibiotic-susceptible E. coli Lac\textsuperscript{+}. The MIC for each population was determined on minimal medium supplemented with low levels of glucose and high levels of lactose. On this medium, the resistant population uses only glucose as a carbon source and grows as a faint lawn, while the susceptible population also consumes lactose and grows as a denser lawn or colonization. In this study, the resistant population was used.

**Preparation of resistant strains**

Ten microlitres of overnight cultures of the antibiotic-resistant strains were inoculated into 1 mL of fresh LB broth supplemented with chloramphenicol or kanamycin and incubated at 37°C under vigorous shaking. After
Antibiotic-resistant strains were prepared in the same volume of PBS medium supplemented or not with IPTG (final concentration 50 μM, unless stated otherwise) to induce the cloned resistance genes. For each set, two mixtures of resistant and susceptible bacteria were prepared, which varied in the number of antibiotic-susceptible cells used. When using E. coli susceptible cells for the test (ΔacrA strain DA34574 for tests with erythromycin or DA5438 for all other antibiotics), 40 μL of the suspension of antibiotic-resistant cells diluted 1:10 (~10^7 cells) was mixed with 10 μL of the undiluted overnight culture of Micrococcus sp. cells or 10 μL of Micrococcus sp. diluted 1:40.

### MIC determination

Each mixture was spread onto half of an M9 agar (supplemented with 0.04% glucose and 0.2% lactose, unless stated otherwise) or LB agar plate (mixtures with E. coli) or Micrococcus sp. susceptible populations, respectively) supplemented or not with IPTG for induction of the cloned resistance genes (50 μM, unless stated otherwise) using pre-wet sterile cotton swabs. Etest strips (bioMérieux) were used to determine the MICs of the 10 previously mentioned antibiotics and mecillinam, clarithromycin and tigecycline. Etest strips were applied onto the plates at the junction between the two spreads and the plates were incubated for 3 days at 37°C in the dark. MIC values were read after each day of incubation.

### Preparation of mixtures of resistant and susceptible bacteria

For each set, two mixtures of resistant and susceptible bacteria were prepared, which varied in the number of antibiotic-susceptible cells used. When using E. coli susceptible cells for the test (ΔacrA strain DA34574 for tests with erythromycin or DA5438 for all other antibiotics), 40 μL of the suspension of antibiotic-resistant cells diluted 1:10 (~10^7 cells) was mixed with 10 μL of the overnight culture of antibiotic-susceptible cells diluted 1:40 (~10^6 cells) or 10 μL of antibiotic-susceptible cells diluted 1:10000 in 1× PBS (~3×10^5 cells). When using Micrococcus sp. susceptible cells for the test (DA34349), 40 μL of the suspension of antibiotic-resistant cells diluted 1:10 was mixed with 10 μL of the undiluted overnight culture of Micrococcus sp. cells or 10 μL of Micrococcus sp. diluted 1:40.
### Antibiotic modification/degradation in the supernatant

Resistant *E. coli* strains used for this test are listed in Table 1. For each antibiotic, two vials containing 2 mL of LB broth supplemented with antibiotics (100 mg/L ampicillin, 50 mg/L fosfomycin, 60 mg/L chloramphenicol, 30 mg/L tetracycline, 250 mg/L erythromycin, 50 mg/L kanamycin, 50 mg/L gentamicin, 50 mg/L tobramycin, 50 mg/L spectinomycin or 50 mg/L streptomycin) were prepared. Media were supplemented with IPTG (50 mg/L) when induction of the cloned resistance gene was required (see Table S2). One microlitre (~10⁶ cells) of pre-culture of the antibiotic-resistant strain to test was inoculated into one of the vials while the second vial was kept sterile and served as a control. Both vials were incubated overnight at 37°C under vigorous shaking. After growth, cultures were centrifuged and the supernatants were filter-sterilized (0.22 μm pore size). Controls and sterilized used media containing antibiotics were serially diluted in a microtitration plate, ensuring that each well always contained 50% of fresh LB broth and 50% of used medium [for this purpose, a stock of used medium was prepared by filter sterilization of an overnight growth of the susceptible strain (DA3438) inoculated into 50 mL of LB broth]. The wells were then inoculated with 1 μL of a 1:1000 dilution (~5×10^7 bacteria) of pre-cultures of antibiotic-susceptible strains DA34574 (ΔacrA strain used for tests with erythromycin) or DA5438 (for all other antibiotics). After 20 h of incubation at 37°C under vigorous shaking, growth of the susceptible strain was monitored by measuring the absorbance at 620 nm (A620) using a Multiskan FC instrument (Thermo Fisher). Growth observed in dilutions of the antibiotic-supplemented media that had been kept sterile or antibiotic-resistant strains used for this test are listed in Table 1. For each antibiotic, the following antibiotics: tetracycline, 3, 5, 7 or 9 mg/L [for resistant strains DA34707 and DA34342 expressing tet(X2) and tet(M), respectively]; chloramphenicol, 8, 10, 12 or 14 mg/L (for resistant strain DA34435 expressing catA1); kanamycin, 4, 6, 8 or 10 mg/L (for resistant strain DA34341 expressing aphA); or ampicillin, 6, 12, 24 or 48 mg/L (for resistant strain DA34348 expressing blaTEM-1A). Plates were incubated overnight at 37°C followed by two additional days at room temperature. Growth of satellite colonies around colonies of resistant cells was monitored every day.

### Results

**IR to several classes of antibiotics**

IR was determined as described in the Materials and methods section. The resistant *E. coli* populations used expressed genetic determinants causing antibiotic resistance by modification or destruction of the antibiotic, by target overproduction, modification or protection, or by antibiotic excretion (Table 2). Genes were cloned into a plasmid and expressed from an IPTG-inducible promoter.

#### Table 2. Resistance genes tested in this study

<table>
<thead>
<tr>
<th>Gene (alternative name) cloned in IPTG-inducible plasmid</th>
<th>Resistance to^a^</th>
<th>Resistance mechanism</th>
<th>Cofactor/other substrate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance to macrolides</td>
<td>ERY, CLR</td>
<td>AB destruction—hydrolyase</td>
<td>H₂O</td>
<td>38</td>
</tr>
<tr>
<td>ere(A)</td>
<td>ERY</td>
<td>target modification (23S rRNA)</td>
<td>—</td>
<td>39</td>
</tr>
<tr>
<td>erm(B)</td>
<td></td>
<td>AB destruction—hydrolyase</td>
<td>H₂O</td>
<td>38</td>
</tr>
<tr>
<td>Resistance to fosfomycin</td>
<td>FOF</td>
<td>AB destruction—glutathione–S-transferase</td>
<td>glutathione</td>
<td>40</td>
</tr>
<tr>
<td>fosA3</td>
<td>FOF</td>
<td>target overproduction</td>
<td>—</td>
<td>41</td>
</tr>
<tr>
<td>murA</td>
<td></td>
<td>AB modification—acyltransferase</td>
<td>acetyl-CoA</td>
<td>42</td>
</tr>
<tr>
<td>Resistance to aminoglycosides</td>
<td>GEN, TOB</td>
<td>AB modification—acyltransferase</td>
<td>acetyl-CoA</td>
<td>42</td>
</tr>
<tr>
<td>aac(3)-Ia</td>
<td>KAN</td>
<td>AB modification—acyltransferase</td>
<td>acetyl-CoA</td>
<td>42</td>
</tr>
<tr>
<td>aac(6′)-Ib-cr</td>
<td>KAN</td>
<td>AB modification—acyltransferase</td>
<td>acetyl-CoA</td>
<td>42</td>
</tr>
<tr>
<td>aph(3′)-Ia (aphA)</td>
<td>STR, SPT</td>
<td>AB modification—acyltransferase</td>
<td>ATP</td>
<td>42</td>
</tr>
<tr>
<td>ant(3′) (aadA2)</td>
<td>SPT</td>
<td>AB modification—acyltransferase</td>
<td>ATP</td>
<td>42</td>
</tr>
<tr>
<td>ant(3′) (aadA5)</td>
<td></td>
<td>AB modification—acyltransferase</td>
<td>ATP</td>
<td>42</td>
</tr>
<tr>
<td>Resistance to tetracyclines</td>
<td>TET, TGC</td>
<td>AB modification—monooxygenase</td>
<td>FAD, NADPH, O₂</td>
<td>43</td>
</tr>
<tr>
<td>tet(M)</td>
<td>TET</td>
<td>target protection (ribosome)</td>
<td>—</td>
<td>28</td>
</tr>
<tr>
<td>tet(G)</td>
<td>TET</td>
<td>efflux pump</td>
<td>—</td>
<td>28</td>
</tr>
<tr>
<td>Resistance to chloramphenical</td>
<td>CHL</td>
<td>AB modification—acyltransferase</td>
<td>acetyl-CoA</td>
<td>28</td>
</tr>
<tr>
<td>catA1</td>
<td></td>
<td>AB modification—acyltransferase</td>
<td>acetyl-CoA</td>
<td>28</td>
</tr>
<tr>
<td>Resistance to β-lactams</td>
<td>AMP, MEC</td>
<td>AB destruction—hydrolyase</td>
<td>H₂O</td>
<td>44</td>
</tr>
</tbody>
</table>

AB, antibiotic; ERY, erythromycin; CLR, clarithromycin; FOF, fosfomycin; GEN, gentamicin; TOB, tobramycin; KAN, kanamycin; STR, streptomycin; SPT, spectinomycin; TET, tetracycline; TGC, tigecycline; CHL, chloramphenicol; AMP, ampicillin; MEC, meccillinam.

The nature of the cloned genes was determined using the Resfinder online database.45

^aOnly the antibiotics tested in this study are indicated.
As expected, IR was observed with the β-lactams (ampicillin and mecillinam) when resistant populations expressing TEM-1A β-lactamase were used (Figure 1, Figure S1 and S2), confirming previous studies (see the Introduction). We also observed IR for erythromycin and clarithromycin [with ere(A), but not erm(B)], tetracycline [with tet(X2), but not tet(M) or tet(G)], tigecycline [with tet(X2)] and chloramphenicol (with catA1).

Figure 1. Indirect resistance (IR). Strains used for this test are listed in Table 1. Ordinate values >1 correspond to IR and indicate that the resistant population allowed growth of the susceptible population at a higher antibiotic concentration compared with when the susceptible population was grown in the presence of an antibiotic-susceptible control population. Values were calculated from MICs scored after 3 days of incubation. Tests with susceptible E. coli (E.co—DA34574 for erythromycin, DA5438 for all other drugs) and Micrococcus sp. (Micro.—DA34349) are in black and grey, respectively. pIP1100 and p147 are plasmids isolated from clinical isolates and carrying ere(A) and catA1, respectively. For each test, two independent biological repeats were performed and similar results were obtained. Additional results and pictures of Etests are presented in Figures S1 and S2. (a) Antibiotics for which IR was not observed. (b) Antibiotics for which IR was observed. FOF, fosfomycin; TOB, tobramycin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; SPT, spectinomycin; ERY, erythromycin; CLR, clarithromycin; TET, tetracycline; TGC, tigecycline; AMP, ampicillin; MEC, mecillinam; CHL, chloramphenicol.
Gram-negative (E. coli) and Gram-positive (Micrococcus sp.) susceptible populations were both protected by resistant E. coli populations (Figure 1). No IR was observed for fosfomycin (with fosA3 and mraA) or for any of the aminoglycosides [with aac(3)-Ia, aac(6’)-Ib-cr, aphA, aadA2 or aadA5], confirming previous observations.4,9 While a strong IR was already visible after 1 day of incubation for β-lactams and chloramphenicol, IR for tetracycline and erythromycin was best visualized after 2–3 days of incubation (the MICs for the susceptible populations reached similar values to those for the resistant populations; Figure S1). For bacteriostatic antibiotics tetracycline, chloramphenicol, erythromycin and clarithromycin, all the susceptible bacteria plated that were affected by IR grew, while at high concentration of the bactericidal antibiotic ampicillin, no or rare colonies of susceptible cells grew compared with the population initially plated (Figure S2; ampicillin >24 mg/L and ampicillin >1 mg/L for E. coli and Micrococcus susceptible strains, respectively). We noticed that all bacteria plated for the bactericidal antibiotic mecillinam grew in our test on M9 medium, which might indicate a bacteriostatic activity of this antibiotic in minimal medium (Figure S2).

To confirm that IR was not the result of our cloning approach, we tested cocultures of susceptible bacteria mixed with E. coli carrying plasmids found in clinical isolates and expressing ere(A) (plasmid pLP11001616) or catA1 (plasmid p147 from isolate 14717). IR was observed with both plasmids, demonstrating that the IR phenotypes described using cloned resistance genes were not artefacts due to artificially high levels of expression (Figure 1 and Figure S2).

**IR is dependent on the level of expression of the resistance marker and the density of the resistant population**

We determined how IR was affected by the level of expression of the resistance determinant and growth of the resistant population. Using tetracycline-resistant populations expressing Tet(X2), growth was examined on M9 plates supplemented with increasing levels of IPTG (to induce different levels of expression of Tet(X2)) or glucose (to vary the density of the resistant population on the plates). IR was dependent on both the level of expression of the resistance marker and the growth or density of the resistant population (Figure 2, Figure S3 and S4). IR was observed when glucose was added at a lower concentration tested (0.008%), despite the absence of noticeable growth of the resistant population. Thus, a very low density of resistant cells was sufficient to protect the susceptible population (Figure 2 and Figure S4).

**Effect of antibiotic-modifying or –degrading enzymes on the concentration of active antibiotic in the extracellular medium**

IR was observed with some antibiotic-modifying or –degrading enzymes, but not with other mechanisms of resistance. Thus, we speculated that IR could be dependent on the ability of the resistance determinants to decrease the concentration of active antibiotic in the medium. To test this hypothesis, we evaluated the concentration of active antibiotic still present in the medium following overnight growth of the resistant strains using a

![Figure 2](image-url). IR is dependent on the level of expression of the resistance marker and on the density of the resistant population. Pictures of Etests are presented in Figures S3 and S4. Resistant populations are E. coli DA34707 [Tet(X2)] and DA34342 [Tet(M)] and the susceptible population is DA5438 (WT E. coli). Plates were incubated for 3 days at 37°C. TET, tetracycline. (a) IR as a function of the level of expression of the resistance markers. Medium was M9 plates supplemented with 0.2% lactose, 0.04% glucose and variable amounts of IPTG. (b) IR as a function of the growth of the resistant population. Medium was M9 plates supplemented with 0.2% lactose, 50 μM IPTG and variable amounts of glucose.
bioassay (see the Materials and methods section). We found a strong correlation between the ability of resistant bacteria to decrease the concentration of active antibiotic in the medium and IR. As expected, resistance determinants that do not modify or degrade antibiotics [Erm(B), MurA, Tet(G) and Tet(M)] had no impact on the concentration of active antibiotic in the medium (Figure S5). Tet(X2), CatA1, Ere(A) and TEM-1A decreased the concentration of tetracycline, chloramphenicol, erythromycin and ampicillin, respectively. No decrease in the concentration of fosfomycin or aminoglycosides was observed with FosA3 or any of the aminoglycoside-modifying enzymes tested (Figure S5). With Ere(A), results were more variable than for other resistance enzymes. We also noted that IR on plates took longer to be detected with Ere(A) than other resistance determinants (IR visible after 3 days, see Figure S1). Slow erythromycin destruction by Ere(A) could explain the variability in the measures and the delayed IR.

**IR affects cells at a distance**

If IR depends on resistance enzymes decreasing the concentration of active antibiotic in the medium, it is expected that IR should affect susceptible cells at a distance, i.e. colonies of resistant cells on plates should allow growth of nearby susceptible cells. This is commonly observed when β-lactamase-producing colonies are surrounded by satellite colonies of β-lactam-susceptible cells. Protection of erythromycin-susceptible bacteria at a distance by Ere(A)-expressing cells was previously observed, but satellite colonies have not been described for other antibiotic resistance determinants. We tested whether satellite colonies could be observed around colonies of cells expressing Tet(X2), Tet(M), AphA, CatA1 or TEM-1A on plates supplemented with increasing concentrations of tetracycline, kanamycin, chloramphenicol or ampicillin (see the Materials and methods section). Examples of satellite colonies are presented in Figure 3. Colonies of antibiotic-resistant cells expressing Tet(X2) or CatA1 allowed growth of nearby susceptible bacteria, indicating that IR could indeed act at a distance. No colonies of susceptible cells grew in areas of the plates for from resistant colonies, indicating that growth of satellite colonies did not result from spontaneous antibiotic degradation in the plates or from spontaneous emergence of antibiotic-resistant mutants among the susceptible population. No satellite colonies were observed around colonies of cells expressing AphA (for kanamycin resistance) or Tet(M) (for tetracycline resistance). The density of susceptible bacteria used in our test (10⁷ cells plated over the entire surface of the plate) only allowed growth of rare, but big, satellite colonies around resistant colonies expressing TEM-1A (for ampicillin resistance).

Compared with satellite colonies around TEM-1A-expressing colonies, which were visible after 2 days of incubation at concentrations ≥32× MIC of ampicillin for the susceptible strain (MIC 1.5 mg/L ampicillin), satellite colonies around colonies expressing Tet(X2) and CatA1 were small and only visible when antibiotic concentrations were close to the MIC for the susceptible population. Furthermore, for tetracycline, satellite colonies required 3 days of incubation to be visible on plates supplemented with 2× MIC for the susceptible strain (MIC 1.5 mg/L). For chloramphenicol, satellite colonies appeared after 2 days on plates supplemented with 2× and 2.5× the MIC for the susceptible strain (MIC 4 mg/L) and after 3 days on plates supplemented with 3.5× the MIC for the susceptible strain. Combined with the delayed IR observed with Tet(X2)- and Ere(A)-producing bacteria compared with TEM-1A-producing bacteria, these results suggest that CatA1, Tet(X2) and Ere(A) were not as efficient as β-lactamases at reducing the concentration of active antibiotics in the medium.

**IR is dependent on high amounts of antibiotics reaching the antibiotic-modifying or -degrading enzymes**

We found that IR was dependent on enzymes efficiently decreasing the concentration of antibiotics in the medium. However, IR and reduction in the concentration of antibiotics in the medium were not observed with all antibiotic-modifying or -degrading enzymes. We speculated that efficient reduction in the concentration of drugs in the medium might depend on whether the intracellular enzymes could reach and modify or destroy large amounts of antibiotics. To test this hypothesis, we compared IR caused by resistant strains expressing ere(A) in different genetic backgrounds (acrA⁺ and acrA⁻). Deletion of the acrA gene increased erythromycin susceptibility (MIC decreasing from 48 to 1.5 mg/L; see Table S2) due to higher intracellular erythromycin concentrations resulting from decreased efflux of the drug following mutation of the AcrAB-ToLC multidrug efflux pump. Thus, we expected Ere(A) to access larger amounts of substrate (erythromycin) and IR to be more pronounced in a ΔacrA mutant than in an acrA⁺ strain. IR was stronger after 2 days in an acrA⁻ than in an acrA⁺ genetic background (Figure 4). Increasing intracellular erythromycin concentration increased IR, revealing that access of the antibiotic-modifying enzyme to its substrate was an important parameter for development of IR.
To our knowledge, IR in the presence of classes of antibiotics confirms the relevance of the IRs observed in our study. Minimants present on plasmids isolated from clinical isolates, observed when resistant strains expressed resistance deter-

germining the concentration of active antibiotics in the extracellular medium. We hypothesized that the ability of antibiotic-modifying or -degrading enzymes to decrease the concentration of drug in the extracellular medium and cause IR was dependent on the intracellular concentration of antibiotic reached, which dictates the amount of drugs the enzyme will be able to modify or degrade. The intracellular concentration of an antibiotic is dependent on both its influx rate through the membranes and its efflux by membrane pumps. Our results with erythromycin when using strains with decreased erythromycin efflux (acrA mutant) are compatible with our hypothesis, since increasing the intracellular concentration of erythromycin by decreasing efflux increased IR. Further support for our hypothesis comes from the observation that antibiotics that allowed IR could enter cells more easily than antibiotics that did not allow IR. Tetracycline and chloramphenicol, for which IR was observed, passively cross the outer membrane using OmpF and PhoE outer membrane porins in E. coli and efficiently diffuse through the lipid bilayer of the inner membrane. On the other hand, fosfomycin and aminoglycosides rely on active import mechanisms for entering the cell and active efflux of aminoglycosides through the AcrD pump was also described. Low influx and/or high efflux translate into low intracellular concentrations of antibiotics and loss of access of the resistance enzymes to their substrates. This would prevent enzymes degrading fosfomycin or modifying aminglycosides from efficiently decreasing the concentration of drug in the extracellular medium and causing IR. In E. coli, fosfomycin is actively transported into the cell by UhpT and GlpT transporters, which are induced by glucose–6-phosphate (G6P) and glycerol–3-phosphate, respectively. E. coli and Enterobacteria Etest contain G6P that should be consumed by the growing resistant population. This would quickly reduce the rate of fosfomycin uptake and might prevent FosA3 from degrading large amounts of fosfomycin and causing IR.

Although our tests were not quantitative, our observations suggest that CatA1, Ere(A) and Tet(X2) were not as efficient at degrading or modifying antibiotics in the medium and causing IR as TEM-1A β-lactamase. We suspect that a better efficiency of β-lactamases at causing IR results from their periplasmic localization and energy-independent hydrolysis mechanism.

**Discussion**

We determined the extent of IR and the general parameters involved in its development. In addition to resistant bacteria expressing β-lactamases, IR was observed with bacteria expressing other antibiotic-modifying or -degrading enzymes in the presence of tetracyclines [tetracycline and tigecycline, Tet(X2) enzyme], chloramphenicol (CatA1 enzyme) and macrolides [erythromycin and clarithromycin, Ere(A) enzyme]. IR was also observed when resistant strains expressed resistance determinants present on plasmids isolated from clinical isolates, confirming the relevance of the IRs observed in our study. To our knowledge, IR in the presence of classes of antibiotics other than β-lactams has not been described to date. The enzymes Tet(X2), CatA1 and Ere(A) are expressed in the cytoplasm and have very different reaction mechanisms and energy dependencies (Table 2). Thus, IR was not limited to β-lactamase-producing bacteria and not linked to the specificities (periplasmic localization and energy-independent hydrolysis activity) of β-lactamases.

IR was observed with antibiotic-modifying or -degrading enzymes, but not with other resistance determinants, correlated with a decrease in the concentration of active antibiotic in the extracellular medium and affected susceptible cells in a species-independent manner. Furthermore, IR affected susceptible cells at a distance and affected all initially plated susceptible cells when bacteriostatic antibiotics were used. Protection at a distance and of all susceptible cells are phenotypes incompatible with mechanisms requiring transfer of genetic material to the suscep-
tible population. The dependency on decreased amounts of active antibiotics in the extracellular medium and the species-independent protection exerted also argues against IR involving cell–cell contact or cell–cell communication. Instead, we suggest that, similarly to what had been proposed for populations expressing β-lactamases, IR results from resistant cells lowering the concentration of active antibiotics in the extracellular medium and allowing susceptible cells to resume growth once the drug concentration falls below their MIC. A mechanism for IR is proposed (Figure 5). Most importantly, we found a strong correlation between IR and resistance enzymes decreasing the concentration of active antibiotics in the extracellular medium.
Furthermore, β-lactamases can also be permanently released extracellularly where they remain active and degrade β-lactam antibiotics. On the other hand, Tet(X2) and CatA1 are sequestered in the cytoplasm and depend on intracellular cofactors and energy for activity, which prevents the release of active enzyme into the medium. Ere(A), which only requires water for catalysis, might remain active in the extracellular medium if released (e.g., following lysis of the resistant cells after prolonged incubation). However, Andremont et al. failed to detect Ere(A) activity in the supernatant of ere(A)-expressing E. coli, revealing that Ere(A) released into the extracellular medium is unlikely to contribute to decreasing the concentration of active erythromycin. Thus, to...
reduce the extracellular concentration of active drugs and cause IR, Tet(X2), CatA1 and Ere(A) must efficiently modify or degrade large amounts of antibiotics present in the cytoplasm, which is affected by the relative rates of influx and efflux of the antibiotics into the cells (Figure 5). Although the IR we describe here might require closer proximity between the susceptible and resistant populations than in the case of β-lactamases, this is most likely irrelevant in biofilms where bacteria are spatially closely related.

We expect that the resistance enzymes to cause IR will vary depending on the bacterial isolate or species expressing it, due to differences in influx and efflux rates of antibiotics. For example, mycobacteria and Pseudomonas aeruginosa have decreased influx rates for numerous antibiotics due to their lack of outer membrane porins allowing diffusion of large molecules. IR will also depend on: (i) presence in the resistant population of additional resistance determinants or mutations affecting influx/efflux of the drug; (ii) activity of the antibiotic-modifying or -degrading enzyme and level of expression of the resistance gene (e.g. variation in promoter strength between species, or gene expressed on plasmids with different copy number); (iii) density of the resistant population and spatial relation between the different populations involved; (iv) activity of the antibiotic (bactericidal, which allows growth of all susceptible cells protected, versus bacteriostatic, which only allows growth of the subpopulation of susceptible cells that survived the initial high concentration of drug); and (v) composition of the environment (e.g. presence of inducers of the fosfomycin transporters, alteration of the negative charge of LPS by bivalent cations modifying outer membrane permeability to numerous drugs, osmolarity-dependent regulation of outer membrane porin expression or induction of multidrug resistance pumps in the presence of bile and fatty acids). In addition to the resistance determinants tested in this study, numerous additional antibiotic-modifying or -degrading enzymes have been described that might be involved in IR. These include, but are not limited to, enzymes modifying or degrading tetracyclines [Tet(X), Tet(X1) and Tet(37)], macrolides [Inu and Mph(A)], fosfomycin (FosB, FosC and FosX), rifamycins (ARR and uncharacterized glycosyltransferases and kinases), daptomycin (hydrolases) and fluoroquinolones (AAC(6′)-Inu and Mph(A)),29 fosfomycin (FosB, FosC and FosX),30 rifamycins (ARR and uncharacterized glycosyltransferases and kinases),31 daptomycin (hydrolases) and fluoroquinolones (AAC(6′)-Inu and Mph(A)).

Our work suggests that coinfecting agents or bacteria from the normal microflora may have a more important negative impact during antimicrobial therapy than previously thought. Whether IR described in this study would also be observed in a biofilm model or during coinfection remains to be determined. If clinically relevant, IR could be prevented by the use of inhibitors of the modifying or degrading enzymes, as described for mixed infections with β-lactamase producers.13,14 While antibiotic resistance genes found in pathogens are widely studied, those found in bacteria isolated from the environment and in commensal or non-pathogenic bacteria remain mostly unknown.18,33–35 Still, drug resistance by modification or destruction is frequently found when sampling environments such as soil, water or the human gut microbiome.10–12 However, additional unknown antibiotic-modifying or -degrading enzymes are expected in those environments (e.g. enzymes specific to non-cultivable organisms or enzymes that are not functional in functional metagenomics studies) and a better understanding of the resistome of non-pathogenic bacteria would be required in order to design practical approaches aimed at detecting and preventing IR.

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Transparency declarations
None to declare.

Supplementary data
Tables S1 and S2 and Figures S1 to S5 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References

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