Anaerobiosis modulates the performance of antimicrobial resistance genes in *Enterobacteriaceae*

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Abstract

Bacteria must face and adapt to a variety of physicochemical conditions in the environment and during infection. A key condition is the partial pressure of oxygen (PO_2), since many colonizable compartments are anaerobic. Enterobacteria comprise frequently resistant pathogens with complex lifestyles, capable of thriving in the (anaerobic) gut, and the environment. Here, we sought to understand if resistance determinants commonly found in enterobacteria can be influenced by oxygen pressure. To do so, we have compared the MIC in aerobic and anaerobic conditions of isogenic *E. coli* strains containing 136 different resistance genes against 9 antibiotic families. Our results show a complex landscape of changes in the performance of resistance genes in anaerobiosis. Certain changes are especially relevant for their intensity and the importance of the antibiotic family, like the large decreases in resistance observed against ertapenem and fosfomycin among bla_{VIM} ß-lactamases and *fos* genes respectively. The pattern of resistance change in anaerobiosis was also conserved in *K. pneumoniae* -although with different intensity. Analyzing other genetic elements of clinical relevance, we observed that pOXA-48 plasmid conferred 4-fold higher ertapenem resistance in anaerobiosis. Last, using a collection of clinical isolates and agar diffusion susceptibility tests, we show that antibiotic susceptibility of multidrug resistant strains differ between aerobic/anaerobic conditions. Our results suggest that anaerobiosis is a relevant aspect to be considered in clinical chemotherapy, as well as in the understanding of the selection and spread of some AMR genes.

43 **Importance**

44 Antibiotic chemotherapy is often based on the results of antibiograms performed in
45 aerobic conditions. Yet many body compartments and infection sites are anaerobic.
46 Enterobacteria are among the deadliest and most antibiotic-resistant pathogens worldwide.
47 They are also commensal inhabitants of our gut, where they thrive in the absence of oxygen.
48 This work shows that antimicrobial resistance genes in enterobacteria can confer different
49 resistance levels in aerobic/anaerobic conditions. Such effect can be important when treating
50 anaerobic infection sites based on antibiogram results. On one hand, the selected therapy can
51 fail in the case of genes that confer higher resistance in anaerobiosis, like $bla_{\text{OXA-48}}$; on the
52 other, successful therapeutic options might be discarded if the gene confers lower levels of
53 resistance in anaerobiosis, like bla_{VIM} carbapenemases. Our results indicate that anaerobic
54 conditions of the site of infection can be an important aspect to consider when treating
55 antimicrobial-resistant bacteria.

56 **Keywords:** Antimicrobial resistance, anaerobiosis, integrons, bla_{VIM}, pOXA-48,
57 fosfomycin

58 Antimicrobial resistance (AMR) is one of the major health concerns of the 21st century
59 globally, according to the World Health Organization (WHO)[1]. Genetic platforms that foster
60 the horizontal transfer of resistance determinants, such as plasmids and integrons[2] play a
61 major role in AMR[3,4]. Plasmid-borne Mobile Integrons (MIs) carry ca. 200 antimicrobial
62 resistance cassettes (ARCs) against various antibiotic families[5], and are extremely prevalent
63 among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*[6,7], pathogens that
64 cause more than 1 million deaths every year[8]. In this work we show that AMR genes may
65 confer different resistance levels in anaerobic conditions, which has potential clinical and
66 eco-epidemiological consequences.

67 Antibiotic resistance is generally measured through diffusion or dilution antibiograms.
68 Agar diffusion-based antibiograms represent a rapid, robust, and comparable measure of
69 resistance, but do not provide precise quantitative data (except when using E-Test strips).
70 Dilution-based antibiograms allow for the determination of the minimal inhibitory
71 concentration (MICs) of an antibiotic[9,10]. Although it can overlook small changes in
72 susceptibility, this technique remains the gold standard in resistance measurement. Despite
73 its general utility and the continuous revisions of clinical breakpoints by *ad hoc* institutes,
74 such as CLSI or EUCAST, the antibiogram does not capture the complex physicochemical
75 conditions encountered by bacteria in different body compartments. A key condition is the
76 local concentration of oxygen. While the partial pressure of oxygen (PO_2) in the respiratory
77 tract reaches 110-150 mmHg (the PO_2 of air is 160 mmHg), most infection sites have lower
78 PO_2 -like blood (40-90 mmHg)- and many are anaerobic, such as the urinary tract (23-45
79 mmHg), abscesses, or deep tissue infections (5-50 mmHg), or the cell interior (PO_2 1-10
80 mmHg)[11–15]. In addition to infection sites, most pathogenic species of enterobacteria colonize
81 the gut, a largely anaerobic compartment (PO_2 of 57-65 mmHg).

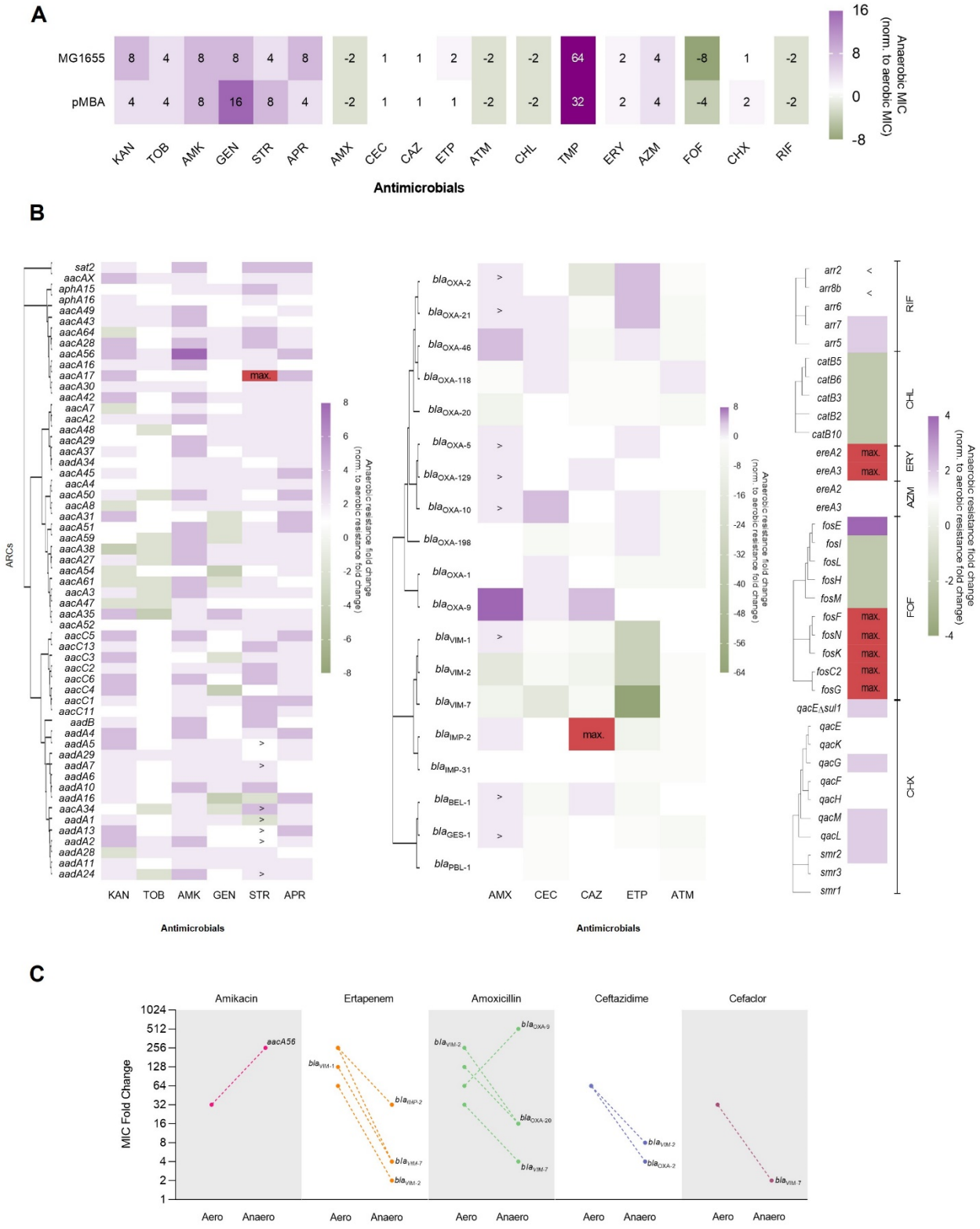
82 From a practical chemotherapeutic perspective, the local oxygen concentration is only
83 taken into account to prescribe molecules active on anaerobic pathogens, and occasionally
84 considering the reduced activity of some antibiotics, such as aminoglycosides, due to an
85 impaired uptake of the antibiotic in anaerobic conditions[14]. In the context of the AMR crisis,
86 we wanted to explore the importance of anaerobiosis in the function of AMR determinants
87 encoded in mobile genetic elements: are these genes equally effective in the presence or
88 absence of oxygen?

89 To characterize the performance of resistance genes, we first need to disentangle it from
90 the effect of anaerobiosis on the host. Hence, we initially compared the susceptibility of *E.*
91 *coli* MG1655 to a set of different antibiotics commonly used in clinical settings under aerobic
92 and anaerobic conditions ($PO_2 \approx 10$ mmHg) (Fig. 1A). We found marked changes in
93 resistance levels between conditions depending on the antimicrobial family tested. In
94 anaerobic conditions, we observed a sharp increase in trimethoprim resistance (64-fold), as
95 well as the expected rise in aminoglycoside resistance (8-fold). In contrast, fosfomycin
96 resistance was 8-fold lower in anaerobiosis.

97 To investigate the impact of anaerobiosis on resistance genes, we used the pMBA
98 collection[16]. It is composed of 136 isogenic strains of *E. coli MG1655,* each containing a
99 particular ARC as the first cassette of a class 1 integron borne by the pMBA plasmid vector.
100 The collection contains resistance genes against 9 antimicrobial families covering a broad
101 variety of mechanisms. We have determined the MIC levels of every member in the collection
102 in the presence and absence of oxygen (Figs. 1B and 1C) (Figs. S1 to S4). As a control, a
103 strain containing the empty pMBA vector was included. The performance of an AMR gene
104 is hence measured as the fold-increase in resistance normalized to the empty pMBA.

105 Additionally, this strain reproduced the effect of anaerobiosis in *E. coli* without pMBA,
106 allowing to rule out confounding effects of the vector (Fig. 1A). Correlations between
107 antimicrobial effects of each ARC in aerobiosis and anaerobiosis are shown in Fig. S5. Our
108 data show that several ARCs varied their antimicrobial resistance effect according to oxygen
109 availability. For example, the aminoglycoside resistance cassette *aacA56* (Figs. 1B and 1C)
110 increases 8-fold the MIC to amikacin in the absence of oxygen. In addition, some families of
111 ARCs showed similar trends in resistance in anaerobiosis: different variants of the metallo-
112 ß-lactamase bla_{VIM} were less efficient in anaerobiosis, reducing their performance against
113 ertapenem between 32 and 64-fold on average (Figs. 1B and 1C). A similar decrease in
114 resistance was observed for several fosfomycin resistance genes (*fos*) in anaerobiosis -with
115 the exception of *fosE,* that showed a 4-fold increase (Figs. 1B). Because both bla_{VIM} and *fos*
116 encode metalloenzymes (that use respectively Zn^{2+} and Mn^{2+} as cofactors) it is tempting to
117 speculate that metal coordination is being affected in anaerobiosis.

118 At least 8 ARCs showed strong changes in their performance in anaerobiosis (fold
119 change ≥ 8-fold) (Fig. 1C). This can be of clinical relevance, as carbapenemases such as
120 bla_{VIMs} and $bla_{\mathrm{IMP\text{-}2}}$, confer lower resistance levels in anaerobiosis. Our data suggests that
121 bacteria carrying these beta-lactamase genes could become susceptible to drugs such as
122 ertapenem in anaerobic body compartments. Yet, the conventional antibiogram would yield
123 an ertapenem resistance phenotype, preventing clinicians from using a possibly successful
124 drug. A similar trend occurs with other beta-lactamases, such as those inactivating
125 aminopenicillins and cephalosporins, with the possible exception of OXA-9-type beta-
126 lactamases.


A
MG1655
pMBA
KAN TOB AMK GEN STR APR AMX CEC CAZ ETP ATM CHL TMP ERY AZM FOF CHX RIF
Antimicrobials
Anaerobic MIC (norm. to aerobic MIC)
B
ARGs
Antimicrobials
Anaerobic resistance fold change (norm. to aerobic resistance fold change)
C
Amikacin
Ertapenem
Amoxicillin
Ceftazidime
Cefaclor
MIC Fold Change
Aero
Anaero

128 Fig. 1. Anaerobiosis affects ARC function in *E. coli*. A) Heatmap showing the fold-change in resistance of *E.*
129 *coli* MG1655 -with and without pMBA- between aerobic and anaerobic conditions against 18 antimicrobials
130 (KAN: kanamycin, TOB: tobramycin, AMK: amikacin, GEN: gentamicin, STR: streptomycin, APR:
131 apramycin, AMX: amoxicillin, CEC: cefaclor, CAZ: ceftazidime, ETP: ertapenem, ATM: aztreonam, CHL:
132 chloramphenicol, TMP: trimethoprim, ERY: erythromycin, AZM: azithromycin, FOF: fosfomycin, CHX:
133 chlorhexidine, RIF: rifampicin). Numbers in each square indicate the fold-change in resistance between
134 anaerobic and aerobic conditions. B) Heatmap of the change in resistance profile of all ARCs in anaerobiosis
135 (fold change in resistance in anaerobiosis compared to aerobiosis) . Purple indicates a that an ARC confers a
136 higher resistance increase (compared to pMBA) in anaerobic conditions than in aerobic, while green color
137 represents a lower resistance increase (compared to pMBA) in anaerobiosis vs. aerobiosis. Red squares depict
138 cases for which a precise MIC could not be determined (strains showing growth at the highest concentration of
139 antibiotic tested) in any of both conditions. Symbols > and < are used to indicate strains that grew at the highest
140 concentration of antibiotic tested in anaerobic or aerobic conditions respectively. A hierarchical clustering tree
141 showing protein sequence similarity is shown in the left side of each panel for (from left to right)
142 aminoglycoside resistance cassettes, beta-lactam resistance cassettes, other ARCs. C) MIC fold change
143 (compared to pMBA) in aerobiosis and anaerobiosis of the subset of ARCs showing at least an 8-fold differences
144 between conditions. Points represent the mode of at least three biological replicates.
145

146 To better understand the clinical importance of these findings, we extended these
147 analysis to other relevant resistance genes and plasmids. Such is the case of *mcr-1* which
148 confers resistance to colistin[17]; the widespread pOXA-48[18] encoding $bla_{\text{OXA-48}}$
149 carbapenemase; or a variant of the natural R388 plasmid, carrying a class 1 integron with 3
150 ARCs ($bla_{\text{VEB-1}}$, *aadB*, and *dfrA5*)[19,20] (Fig. 2A). The resistance profile of hosts with pOXA-
151 48 and R388 is modified in the absence of oxygen. pOXA-48 confers clinical resistance to
152 ertapenem in anaerobiosis, whereas the same *E. coli* is considered susceptible to this
153 antibiotic under aerobic conditions. This exemplifies how conventional susceptibility testing
154 procedures could result in the election of an inappropriate therapy. An analogous

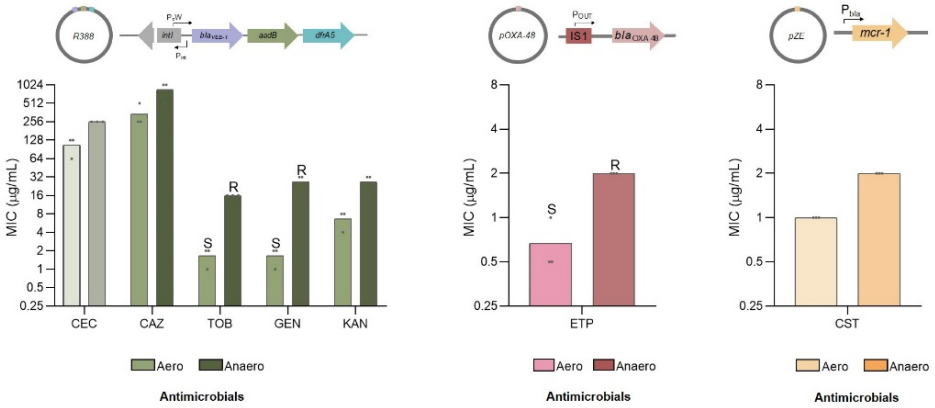
155 phenomenon is observed in R388-containing *E. coli* increasing its resistance to several
156 aminoglycosides and the beta-lactam antibiotic ceftazidime, due to gene $bla_{\text{VEB-1}}$. The
157 resistance profile of *mcr-1* did not show relevant changes (≥ 4-fold change) in anaerobic
158 conditions.

159 Because resistance genes are often borne on mobile genetic elements, we sought to
160 explore if our results were true in other enterobacteria. To do so, we selected the ARCs
161 showing ≥ 8-fold change between conditions and characterized them in *Klebsiella*
162 *pneumoniae* ATCC23357 (Fig 2B and Fig S6). Our data shows that the phenomenon is
163 generally conserved: the trends in resistance changes are similar between species, although
164 there are differences in the magnitude of the change, depending on the antibiotic (Fig 2B).
165 The only exception was *aacA56* that did not increase resistance to amikacin in anaerobiosis
166 in *K. pneumoniae*.

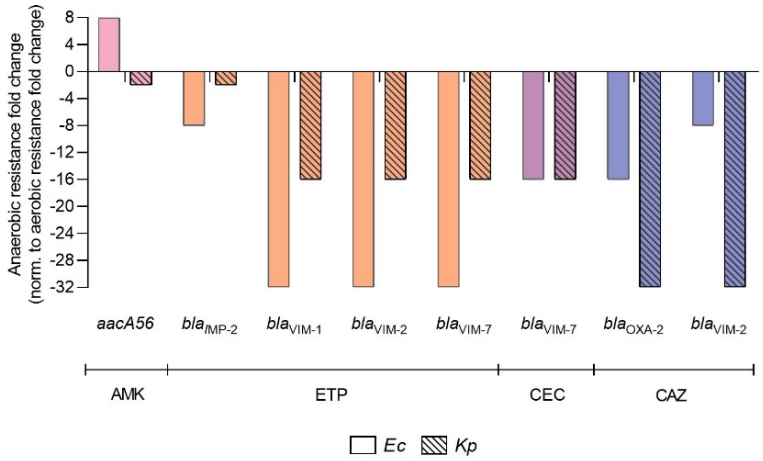
167 Finally, we wanted to address if anaerobiosis could change the resistance profiles of
168 clinical isolates that contain multiple resistance genes. To test this, we used a well-
169 characterized collection of multi-resistant *E. coli* and *K. pneumoniae* isolates obtained from
170 hospitalized patients through the R-GNOSIS project[21–23] (Table S1). We performed diffusion
171 antibiograms against 21 compounds in aerobic and anaerobic conditions (Fig 2C) and
172 observed notable differences in several isolates across a range of compounds -including
173 aminoglycosides, beta-lactams, phenicols, and fosfomycin among others. This supports that
174 our results with laboratory strains are translatable to the clinical setting. It is worth noting

that some of these clinical isolates exhibit differences in their resistance profiles against last-resort beta-lactams such as ertapenem or imipenem depending on oxygen availability.

A



B



C

	EC05		EC10		EC14		Kpn04		Kpn13		Kpn21	
	Aero	Anaero	Aero	Anaero	Aero	Anaero	Aero	Anaero	Aero	Anaero	Aero	Anaero
AMX	6^{R}	6^{R}	6^{R}	6^{R}	6^{R}	6^{R}	6^{R}	6^{R}	6^{R}	6^{R}	6^{R}	6^{R}
AMC	28^{S}	26^{S}	23^{S}	21^{S}	25^{S}	24^{S}	23^{S}	22^{S}	23^{S}	24^{S}	27^{S}	26^{S}
ATM	16^{R}	15^{R}	22^{ATU}	18^{R}	11^{R}	12^{R}	10^{R}	6^{R}	17^{R}	13^{R}	12^{R}	6^{R}
CEF	12	11	6	6	6	6	6	6	6	6	6	6
FOX	27^{S}	26^{S}	26^{S}	20^{S}	26^{S}	24^{S}	11^{R}	17^{R}	24^{S}	27^{S}	19^{S}	28^{S}
CTX	23^{S}	22^{S}	14^{R}	11^{R}	10^{R}	10^{R}	6^{R}	6^{R}	12^{R}	12^{R}	10^{R}	6^{R}
FEP	28^{S}	25^{ATU}	18^{R}	14^{R}	15^{R}	14^{R}	14^{R}	12^{R}	18^{R}	18^{R}	15^{R}	13^{R}
ETP	35^{S}	34^{S}	30^{S}	27^{S}	31^{S}	28^{S}	29^{S}	29^{S}	30^{S}	36^{S}	30^{S}	35^{S}
IMP	30^{S}	33^{S}	30^{S}	30^{S}	30^{S}	25^{S}	32^{S}	38^{S}	29^{S}	35^{S}	32^{S}	37^{S}
STR	15	6	11	6	14	6	6	6	6	6	6	6
GEN	17^{S}	10^{R}	8^{R}	6^{R}	11^{R}	6^{R}	20^{S}	17^{S}	17^{S}	16^{R}	19^{S}	18^{S}
AMK	18^{S}	11^{R}	19^{S}	12^{R}	18^{S}	11^{R}	23^{S}	19^{S}	21^{S}	20^{S}	22^{S}	20^{S}
TOB	17^{S}	10^{R}	6^{R}	6^{R}	16^{S}	10^{R}	20^{S}	17^{S}	18^{S}	18^{S}	19^{S}	18^{S}
KAN	17	11	14	10	19	11	22	20	21	20	22	20
FOF	40^{S}	51^{S}	37^{S}	32^{S}	36^{S}	32^{S}	26	30	25	30	27	31
CIP	13^{R}	9^{R}	35^{S}	31^{S}	19^{R}	14^{R}	16^{R}	11^{R}	22^{ATU}	23^{ATU}	28^{S}	32^{S}
CHL	30^{S}	31^{S}	31^{S}	32^{S}	31^{S}	28^{S}	19^{S}	25^{S}	30^{S}	33^{S}	10^{R}	10^{R}
TET	25^{S}	28^{S}	29^{S}	27^{S}	6^{R}	6^{R}	19^{S}	20^{S}	28^{S}	31^{S}	13^{R}	9^{R}
TMP	26^{S}	26^{S}	30^{S}	30^{S}	25^{S}	25^{S}	6^{R}	6^{R}	6^{R}	6^{R}	6^{R}	6^{R}
RL	6	15	6	11	6	6	6	6	6	6	6	6
SXT	32^{S}	32^{S}	34^{S}	37^{S}	24^{S}	24^{S}	6^{R}	6^{R}	6^{R}	6^{R}	6^{R}	6^{R}

177 Fig. 2. Anaerobiosis modulates antimicrobial susceptibility across different species. A) MIC of *E. coli* MG1655
178 harboring clinically other relevant resistance genes and plasmids, against several antimicrobials. Bars represent
179 the mean of three biological replicates (black dots) for each strain. Light and dark colors represent aerobic and
180 anaerobic conditions respectively. S and R letters stand for susceptible and resistant according to EUCAST
181 clinical breakpoints. A scheme illustrating the relevant genetic background of each gene is displayed above each
182 graph. B) Graph showing the ratio between the fold-change in MIC (compared to pMBA) in anaerobiosis over
183 aerobiosis in *E. coli* and *K. pneumoniae*. Oxygen concentration affects ARCs in a similar way in both species.
184 MIC fold change is calculated as the mode of at least six biological replicates. C) Table showing the diameter
185 of halos in agar diffusion susceptibility tests for clinical isolates from the R-GNOSIS project. Cells shaded in
186 purple indicate a transition from susceptible to resistant according to EUCAST clinical breakpoints. Data in
187 bold indicates notable changes in inhibition halo diameters (≥ 5 mm.), between aerobic and anaerobic
188 conditions. TOB: tobramycin, GEN: gentamicin, ETP: ertapenem, STR: streptomycin, AMK: amikacin, FOF:
189 fosfomycin, AMX: amoxicillin, AMC: amoxicillin-clavulanic acid, ATM: aztreonam, FOX: cefoxitin, CTX:
190 cefotaxime, FEP: cefepime, IMP: imipenem, GEN: gentamicin, KAN: kanamycin, CIP: ciprofloxacin, CHL:
191 chloramphenicol, TET: tetracycline, TMP trimethoprim, RL: sulphamethoxazole, STX:
192 trimethoprim/sulfamethoxazole.
193
194 Our findings suggest that oxygen availability plays a modulatory role in the performance of
195 AMR genes. This phenomenon has significant implications both in clinical practice and
196 ecological studies. While the MIC is widely used as the gold standard for designing antibiotic
197 treatments, its representativity has been questioned due to the limited complexity of the
198 technique compared to the variety of environments where infections occur[24]. Our results
199 suggest that a bacterium may transition from being resistant in aerobic environments to
200 susceptible in anaerobic conditions, depending on its resistome. This highlights the
201 possibility that successful therapies might be ruled out based on aerobic MIC results. The
202 opposite is also true: determinants like *aacA8*, or $bla_{\text{OXA-48}}$ exhibit higher resistance levels in

203 the absence of oxygen, so standard treatments may not be effective against infections
204 developing in anaerobic microenvironments. Hence, the most clinically relevant result of this
205 work is that anaerobiosis generally reduces antibiotic susceptibility, but the rate of this
206 change strongly differs for different genes, and even within gene families; additionally, in
207 some cases antibiotic susceptibility can increase. Our results have also other implications:
208 the elusive problem of diversification of ARGs might have originated from the selection of
209 enzymes in different compartments, anaerobic (as the intestine or the infection site), or
210 aerobic (external environment, surface of respiratory tract, water, soil, sewage). Also, from
211 an ecological perspective, this shift in performance could influence the prevalence and spread
212 of genes in clinical settings. For instance, while $bla_{\text{OXA-48}}$ is one of the most widespread
213 carbapenemases in clinical settings across Europe, some variants of bla_{VIM} are extremely
214 rare. These genes show opposite behaviors in anaerobiosis.

215 The evolutionary success of an AMR genes relies on a variety of factors, of which the
216 resistance level and the biological cost in the absence of antibiotics are arguably the most
217 important ones[25]. Considering the relationship between function and oxygen availability as
218 an additional parameter could better inform strategies to control the spread of resistance. This
219 study could provide the basis for future investigations and improvements in the design of
220 more effective and targeted antibiotic treatments.

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286

287

288 **Methods**

289

290 *Strain collection and culture conditions.*

291 *E. coli* MG1655 and *K. pneumoniae* laboratorial strains were used for this study. These strains
292 are part of the pMBA collection of integron antimicrobial resistance cassettes[16] (see Table
293 S2). Bacterial strains were cultured at 37ºC in Müller Hinton (MH; Oxoid, UK) liquid
294 medium and MH agar (1,5%) solid medium (BD, France). Zeocin (Invivogen, USA) was
295 added at 100 µg/mL to maintain pMBA plasmid collection. Additionally, MG1655 containing
296 clinically relevant plasmids (R388, *mcr-1* containing pZE and pOXA-48) were also used in
297 this study together with *E. coli* and *K. pneumoniae* clinical strains retrieved from colonized
298 patients in a university hospital in Madrid described in the R-GNOSIS project[21] (see Table
299 S1 and S2). Liquid cultures were incubated in an Infors Multitron shaker at 200 rpm (Inforns
300 HT, Swiss) unless otherwise stated.

301

302 *Antimicrobial susceptibility testing.*

303 Minimal inhibitory concentrations (MICs) of each pMBA derivative against selected
304 antimicrobial molecules (for a list of compounds see Table S3) were confirmed by broth
305 microdilution according to the CLSI guidelines[265] with some modifications for anaerobic
306 MIC testing. Briefly, 10^5 colony-forming units (UFCs) were inoculated in 200 µl of fresh
307 MH with doubling dilutions of each selected antibiotic in 96 well-plates (Nunc) and
308 incubated overnight at 37ºC in static conditions. 96 well-plates were incubated inside
309 hermetic recipients where anaerobe container system sachets (BD, US) were deposited to
310 generate the anaerobic atmosphere (less than 1% of O_2, equivalent to about PO_2 10 mmHg).

311 The MIC was established as the lowest concentration in which growth could not be observed.
312 MICs were measured at least in three biological replicates for each antibiotic and strain. MICs
313 of fosfomycin were confirmed by agar dilution in anaerobic conditions. MICs were
314 interpreted using the clinical breakpoints for *Enterobacteriales* defined by the European
315 Committee on Antimicrobial Susceptibility Testing (EUCAST). Additionally, we assessed
316 the antimicrobial resistance of certain clinical isolates using disc diffusion tests in MH-agar
317 broth (for a list of antimicrobial discs see Table S4).