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A large self-transmissible resistance plasmid from Nigeria contains genes that ameliorate a carrying cost

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Antimicrobial resistance is rapidly expanding, in a large part due to mobile genetic elements. We screened 94 fecal fluoroquinolone-resistant *Escherichia coli* isolates from Nigeria for six plasmid-mediated quinolone resistance (PMQR) genes. Sixteen isolates harbored at least one of the PMQR genes and four were positive for *aac-6-*lb-cr**. In one strain, *aac-6-*lb-cr** was mapped to a 125 Kb self-transmissible IncFII plasmid, pMB2, which also bears *bla*_{CTX-M-15}, seven other functional resistance genes and multiple resistance pseudogenes. Laboratory strains carrying pMB2 grew faster than isogenic strains lacking the plasmid in both rich and minimal media. We excised a 32 Kb fragment containing transporter genes and several open-reading frames of unknown function. The resulting 93 Kb mini-plasmid conferred slower growth rates and lower fitness than wildtype pMB2. Trans-complementing the deletion with the cloned *sitABCD* genes confirmed that they accounted for the growth advantage conferred by pMB2 in iron-depleted media. pMB2 is a large plasmid with a flexible resistance region that contains loci that can account for evolutionary success in the absence of antimicrobials. Ancillary functions conferred by resistance plasmids can mediate their retention and transmissibility, worsening the trajectory for antimicrobial resistance and potentially circumventing efforts to contain resistance through restricted use.

Ciprofloxacin was introduced into clinical medicine in the 1980s. It however only became affordable, and therefore widely available in Nigeria in the 2000s, once its patent expired. Fluoroquinolone resistance subsequently emerged in Nigeria¹ and poses an enormous clinical challenge because ciprofloxacin and other orally active quinolones represent the last accessible therapeutic option for many infected patients. High-level fluoroquinolone resistance is most commonly attributable to multiple mutations in the quinolone-resistance-determining regions of quinolone targets *gyrA* and *parC*. In non-mutator *E. coli* strains, the mutation rate is 10⁻⁹ or lower so that step-wise mutation to resistance is predicted to be slow. Resistance evolved quickly in Nigeria, as in many other parts of the world, supporting the idea that low-level resistance mechanisms may help to protect susceptible strains until they accumulate the 2–4 mutations required for high-level resistance². We have previously shown that early quinolone resistant isolates showed elevated efflux capacities¹ but the contribution of plasmid-mediated quinolone resistance (PMQR) in Nigeria and other African countries has been investigated only minimally.

Plasmid-encoded resistance genes most significant in quinolone resistance can be grouped into three functional categories. The *qnr* genes, suspected to be the most common PMQR genes globally, encode members of a pentapeptide-repeat family. They confer resistance through direct binding of their gene products to the quinolone targets and thus prevent interactions between the drug and the target enzyme^{3,4}. A second category of quinolone-resistance genes, *qepA* and *oqxA*, encode efflux pumps. QepA is a member of the 14-transmembrane-segment major facilitator family of transporters and functions as a proton antiporter efflux pump system that is especially efficient at exporting quinolones but which can also export other antimicrobials

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Plasmid-mediated quinolone resistant gene	Individuals	Strains
	Unique individuals carrying ciprofloxacin non-susceptible strains	Total number of ciprofloxacin non-susceptible strains (n = 94)
<i>qnrA</i>	0	0
<i>qnrB</i>	0	0
<i>qnrS</i>	2	2
<i>qepA</i>	2	2
<i>oqxAB</i>	8	9
* <i>aac(6′)-Ib</i>	26	37
<i>aac(6′)-Ib-cr</i>	2	4

Table 1. PMQR genes identified by PCR in ciprofloxacin non-susceptible *Escherichia* isolates. *Includes the PMQR *aac(6′)-Ib-cr* as well as non-ciprofloxacin resistance conferring *aac(6′)-Ib*.

including erythromycin, acriflavine, and ethidium bromide. The plasmid-encoded efflux pump, encoded by the *oqxAB* genes, is a member of the resistance-nodulation-division family of multidrug efflux pumps and was first identified as conferring resistance to the porcine growth enhancer quinoxaline-di-N-oxide, a compound that also inhibits DNA synthesis⁵. *oqxAB*, can also confer resistance to chloramphenicol in addition to the quinolones nalidixic acid and ciprofloxacin^{5,6}. The *oqxA* gene appears to be rare among isolates identified in Denmark, Sweden, and South Korea. Recent reports suggest that it is common in South Africa and potentially other parts of the continent but there are few studies performed in sub-Saharan Africa which have sought these genes^{5,7–11}.

The third and final PMQR category contains only one member. *aac(6′)-Ib-cr* is an aminoglycoside acetyltransferase¹². The standard *aac(6′)-Ib* allele confers aminoglycoside resistance but two SNPs generate an *aac(6′)-Ib-cr* allele that confers resistance to ciprofloxacin and norfloxacin, which have unsubstituted piperazinyl groups, but not to other quinolones¹². The *aac(6′)-Ib-cr* allele was initially identified by Robicsek *et al.* from clinical *E. coli* isolates originating in China⁴. Like the other PMQR genes, with the exception of *oqxA*, *aac(6′)-Ib-cr* has previously been identified in isolates from West Africa¹³. On its own, *aac(6′)-Ib-cr* confers only low-levels of resistance to ciprofloxacin but the gene enables target-site mutants to survive at drug concentrations achieved in the clinic. Additionally, *aac(6′)-Ib-cr* is frequently found on plasmids with other resistance genes including *qnr* genes, and *qepA*, as well as β -lactamases^{2,4}.

Although the aforementioned genes have been found throughout the globe, there remains a dearth of information on the nature of quinolone resistance in Africa and the relative importance of PMQR genes on the continent. Because PMQR genes, as their name suggests, are plasmid-borne, the mobile elements that transmit them have the propensity to bring in other resistance genes and/or non-resistance characteristics, which in turn could influence strain survival, virulence and transmissibility. The selective success of some *E. coli* lineages is attributed fully or in part to resistance gene-bearing elements, which¹⁴ have also been known to confer adherence, virulence, bacteriocin immunity and evolutionary fitness^{14–16}.

We recently examined antimicrobial resistance in *Escherichia coli* from mother-infant pairs in Nigeria and found that 94 of 1,098 (8.6%) of the isolates were fluoroquinolone resistant^{17,18}. We sought to determine the proportion of these strains carrying PMQR genes and the identity of these genes. While we have previously described a plasmid carrying *qnrS1* from a Nigerian isolate¹⁹, no local information is available about the context of *aac(6′)-Ib-cr*. We therefore screened for *aac(6′)-Ib-cr* in this recently obtained epidemiological strain collection and then sequenced and characterized a *aac(6′)-Ib-cr*-bearing plasmid identified from one of the strains.

Results

Plasmid-encoded quinolone resistance genes in fluoroquinolone resistant isolates from Nigeria. Of 1,098 fecal *E. coli* isolates originally recovered from 134 mother-infant pairs, 94 (8.6%) were ciprofloxacin-resistant and were screened for six PMQR genes. The PCR screen initially revealed that *aac(6′)-Ib* was common among ciprofloxacin resistant isolates with 37 (39.3%) being positive for the gene as shown in Table 1. However, upon *Bst*I restriction analysis necessary to confirm the ciprofloxacin resistance-conferring allele, only four of the isolates with *aac(6′)-Ib* genes (10.8%) proved to be the *aac(6′)-Ib-cr* allele (Table 1). *qnrA* and *qnrB* were not detected at all whilst *qnrS1*, which we have previously detected in a different strain set from Nigeria¹⁹, was found in two of the isolates. Overall, *oqxAB* was detected in nine isolates (including two that were positive for *aac(6′)-Ib-cr*) and was therefore the most common PMQR recorded but *aac(6′)-Ib-cr* was the most common quinolone-specific mechanism (Table 1). We therefore went on to characterize M63c, one of the strains bearing *aac(6′)-Ib-cr* alleles.

Strain M63c was mucoid on MacConkey and eosin methylene blue plates with an API 20E profile pointing to ‘group C *E. coli*’, which are now classified as *Escherichia fergusonii*. We amplified and sequenced the 16S rRNA gene of M63c and found the sequence (Accession number SUB5013267) was 99% identical to that of *Escherichia fergusonii* type strain ATCC 35469 (Accession number NR_074902.1). However, Maheux *et al.*²⁰ reported that the *adk*, *gyrB* and *recA* genes were more useful for speciating *Escherichia* spp. than 16S rRNA. Alleles of those genes carried by M63c were identical to *E. coli* alleles. We additionally sequenced *fumC*, *icd*, *mdh* and *purA* genes of the strain and multilocus sequence typed the isolate as an *E. coli* ST167 strain. *E. coli* ST167 strains are encountered around the globe, frequently multiply resistant and often expressing extended-spectrum β -lactamases (ESBLs)²¹. We extracted a large plasmid DNA from strain M63c and electroporated it into DH5 α cells (DH5 α E from Thermo Scientific). Transformants were obtained on neomycin (50 mg/ml) plates but not on ciprofloxacin (1 mg/ml).

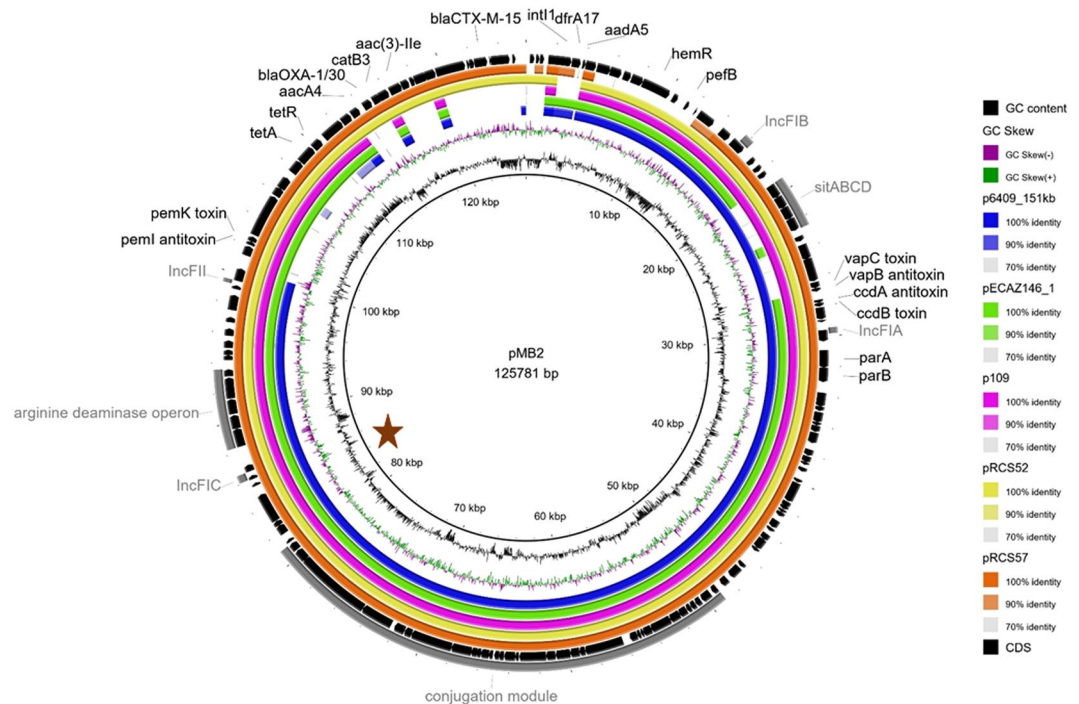


Figure 1. Circular plot illustrating the general features of pMB2. The figure shows the organization of pMB2 ORFs in black and a BLASTn comparison between pMB2 and the five best hits *E. coli* plasmids retrieved from NCBI (from the innermost to the outermost circles, plasmids p6409, pECAZ146_1, p109, pRCS52 and pRCS57 with the GenBank accession numbers CP010372.1, CP018990.1, CP023372.1, LO017736.1 and LO017738.1, respectively). A brown star marks the likely location of the origin of replication.

Each transformant showed reduced susceptibility (but not full resistance) to ciprofloxacin and nalidixic acid as well as resistance to tetracycline, ampicillin, neomycin/kanamycin, trimethoprim and sulphonamides but not chloramphenicol. All transformants had identical plasmid DNA *Bam*HI, and *Sal*II restriction profiles and susceptibility patterns and carried a single large plasmid, which we termed pMB2.

Sequence of Plasmid pMB2. pMB2 was Sanger-sequenced and shot-gun assembled into a 125,782 base pair circular replicon (Genbank Accession number MK370889). pMB2 has a G + C content of 51.73% (Fig. 1), which is comparable to that of the *Escherichia coli* chromosome²². A maximal number of 323 ORFs of approximately 100 or more base pairs in length were predicted but additional evidence supports only 145 of these as encoding real or hypothetical genes. The plasmid contains a full IncFII-type conjugation region of 32 genes including all 25 of the consecutive transfer region genes with known functions in the conjugation process²³.

In silico replicon typing revealed that the Plasmid Sequence Type of pMB2 is [F36:A4:B1]. The pMB2 plasmid carries replication-associated proteins associated with IncFIA, IncFIB, IncFIC and IncFII plasmids. The IncFIC RepA1 protein is coded by an ORF at 86,659 bp on the plasmid, which is close to the major shift in G + C skew seen at 81,000 bp and likely marking the origin of replication (Fig. 1). pMB2 is rich in mobility genes with at least 26 transposases or transposase-like ORFs, as well as up to six other integrases and recombinases encoded. Almost all functional units on the plasmid are flanked by insertion sequences. pMB2 carries genes encoding several toxin-antitoxin systems *ccdA/ccdB*, *vapB/vapC* and *pemI/pemK*. It also carries genes encoding the SOS response inhibiting proteins PsiA and PsiB. Between positions 88,324 and 93,566 is a complete, six gene arginine deaminase operon, which could potentially catabolize arginine to ammonia.

pMB2 is predicted to encode at least three transport systems. The EamA-like domain-containing drug/metabolite and carboxylate/amino acid/amine transporter (Pfam00892; located at 107699–108583), is found in a variety of integral membrane proteins; there is no known function for these genes but they could possibly contribute to antimicrobial resistance. A hypothetical divalent ion transport system (located between 10,045 and 7,247, on the complementary strand) is composed of two proteins coded in an operon: conserved hypothetical pMB2_00012 a (pfam10670)²⁴ and HmuR/hemR, which is a Ton-B dependent hemoglobin-hemin receptor localized to the bacteria's outer membrane²⁵. The third transport system, and the best studied, is encoded by *sitABCD* located on the forward strand of the plasmid between 19,195 and 22,644. SitABCD form an effective ferrous iron uptake system earlier described in *Salmonella enterica* and *Shigella*^{26,27}. SitA is a periplasmic binding protein, SitB is the ATP-binding component, SitC functions as a permease and SitD is the inner membrane component of the system.

pMB2 bears eight genes conferring resistance to six different drug classes (Fig. 2). An IS26-composite transposon flanks an incomplete class I integron carrying a *dfrA17* gene (encoding resistance to trimethoprim) and an incomplete *aadA5* gene. The tetracycline resistance gene *tetA* (alongside *tetR*, encoding the repressor of the tetracycline resistance element) is located within the remnants of a Tn1721 transposon. Next to this region, another

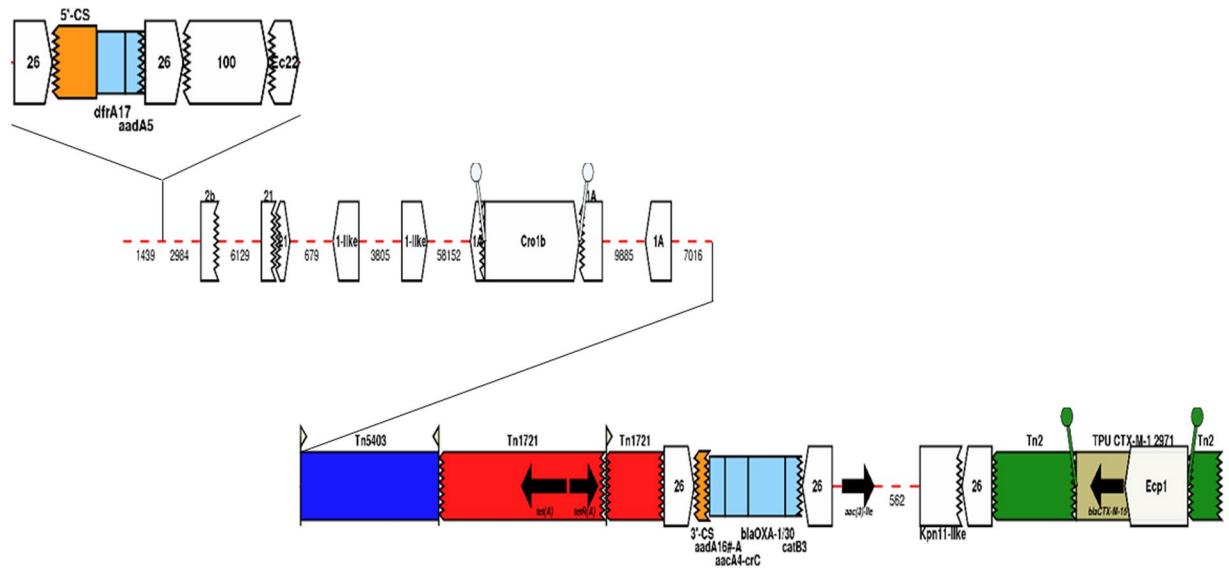


Figure 2. Genetic context of the pMB2 antimicrobial resistance region. Arrows indicate the direction of transcription for genes. Gene cassettes are shown by pale blue boxes, the conserved sequences (5' and 3'-CS) of integrons as orange boxes and insertion sequences as white block arrows labelled with the IS number/name, with the pointed end indicating the inverted right repeat (IRR). Unit transposons are shown as boxes of different colors and their IRs are shown as flags, with the flat side at the outer boundary of the transposon. Direct repeats are shown as 'lollipops' of the same color. The zig-zag line indicates which end of the feature is missing. Gaps > 50 bp are indicated by dashed red lines and the length in bp given.

IS26-composite transposon was identified, carrying an incomplete class I integron with a $[aadA16|aac(6')-Ib-cr|bla_{OXA-1/30}|\Delta catB3]$ gene cassette array encoding resistance to aminoglycosides, fluoroquinolones, beta-lactams and phenicol. As stated previously, the $aac(6')-Ib-cr$ gene, also named $aacA4-crC$, confers resistance to aminoglycosides and the broad-spectrum antimicrobial ciprofloxacin due to a T to C mutation at nt 283 and a G to T mutation at nt 514. Following bla_{OXA-1} is $catB3$, which normally inactivates chloramphenicol through acetylation²⁸ but which is not active in pMB2 due to a truncated cassette region. Next to the IS26-composite transposon is an $aac(3)-II$ gene, conferring gentamicin resistance. Finally, a transposition unit carrying $bla_{CTX-M-15}$, which confers resistance to oxyiminocephalosporins, and $ISEcp1$ was found within the remnants of a Tn2 transposon. This insertion unit is flanked by 5-bp direct repeats (5'-TATGA-3'), suggesting the *en bloc* transposition of these elements within the Tn2 transposon (Fig. 2).

pMB2 is similar to many other resistance plasmids in the database that have been isolated from around the world. Figure 1 compares the sequences of four of these plasmids with pMB2 illustrating that the plasmids are almost completely conserved and almost perfectly syntenic. In particular, conjugation and arginine deaminase genes are highly conserved among the plasmids whereas their resistance regions are expectedly the most variable. Only three of the plasmids carry all the putative transport genes, however all of them have at least two of them.

pMB2 is self-transmissible. While the plasmid as a whole is replete with transposable elements and insertion sequences, and contains multiple antimicrobial resistance genes, some no longer functional, others redundant, the conjugation region of pMB2 is not interrupted. The transfer genes of pMB2 are organized similarly to those of the F plasmid²⁹ and identical to those on the other five plasmids we compared with pMB2 in Fig. 1. Conjugation from DH5 α (pMB2) to EC1502 (rifampicin resistant) on solid media occurred at the rate of 1.8×10^{-5} to 8×10^{-6} .

Three other *Escherichia* isolates from the individual from whom strain M63c was isolated had been archived along with two isolates from her infant¹⁷. As shown in Table 2, strain M63c was the only quinolone-non-susceptible isolate of this set. We screened all six isolates for the presence of the $aac(6')-Ib-cr$ allele and pMB2 and found that they were present in strain M63b, as well as original source strain M63c. While they differed in quinolone resistance, strain M63b and M63c shared otherwise identical susceptibility patterns. We sequenced quinolone resistance determining regions of *gyrA* and *parC* in both strains and found that quinolone-susceptible strain M63b contained no SNPs whilst resistant strain M63c carried Ser83Leu and Asp87Asn mutations in *GyrA* and Ser80Ile, Glu8Val mutations in *ParC*. These findings are consistent with reports that demonstrate that those SNPs are sufficient for high-level fluoroquinolone resistance and $aac(6')-Ib-cr$ allele is insufficient to confer clinically significant ciprofloxacin resistance but augments resistance conferred by other mechanisms^{12,30}. The presence of the plasmid in two different *Escherichia* strains from the same host strongly suggests *in vivo* transmission.

pMB2 does not have a carrying cost. We hypothesized that the large size of pMB2 would slow the growth of laboratory strain DH5 α . In rebuttal of our hypothesis, DH5 α carrying pMB2 grew at rates that were comparable to or faster than the plasmid-free strain. This occurred in Luria broth (LB) (Fig. 3) as well as in nutrient broth,

	Zone diameters (mm) and CLSI interpretations								Minimum inhibitory concentrations ($\mu\text{g/mL}$)		
	C	S	NA	SUL	AMP	CIP	TE	W	NA	CIP	KM
DH5 α E	30S	23S	20S	50S	40S	42S	34S	22S	12	0.023	<7.5
DH5 α E (pMB2)	30S	22S	16I	44S	6R	34S	9R	6R	16	0.094	>30
C600	24S	17S	6R	36S	23S	26S	22S	30S	>256	0.25	<7.5
M63a	21S	14I	22S	6R	6R	39S	6R	6R	6	0.023	<7.5
M63b	22S	13I	21S	6R	6R	42S	7R	6R	6	0.016	<7.5
M63c	21S	10R	6R	6R	6R	6R	6R	6R	>256	>32	>30
M63e	21S	14I	21S	6R	6R	31S	6R	6R	4	0.023	<7.5
C63a	21S	14I	22S	24S	15I	42S	17I	24S	4	0.023	<7.5
C63c	22S	16S	25S	30S	19I	38S	21S	25S	4	0.023	<7.5

Table 2. Antimicrobial susceptibility profiles of *Escherichia* isolates from the mother-infant pair from whom plasmid pMB2 were recovered and DH5 α with and without pMB2. Data are zone diameters in mm and CLSI interpretations (R = resistant, I = intermediate; S = sensitive) and minimum inhibitory concentrations. A nalidixic acid-resistant derivative of *E. coli* C600 was used as a quinolone-resistant control. Key Chloramphenicol (C), Streptomycin (S), Nalidixic Acid (NA), Sulfonamide (S), Ampicillin (AMP), Ciprofloxacin (CIP), Tetracycline (TE), Trimethoprim (W); Resistant (R), Susceptible (S), Intermediate (I). Strains M63a-e are from a mother enrolled in the study of Odetoyin *et al.*¹⁷ and strains C63a and c are isolates from her child.

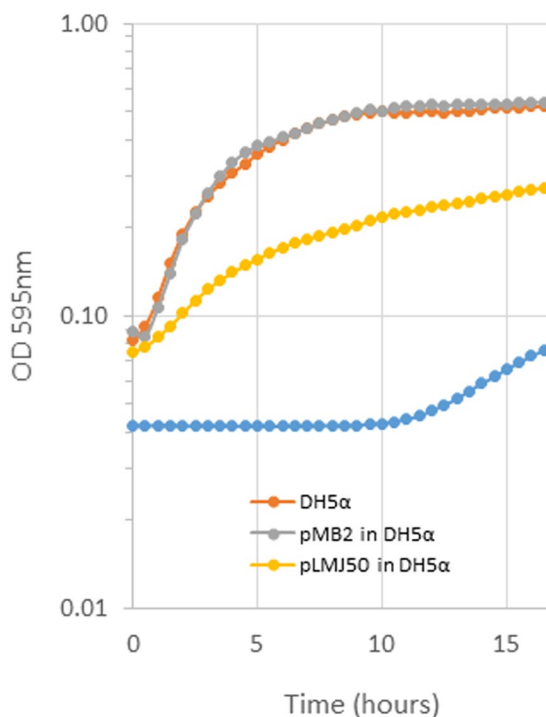


Figure 3. 18 h growth time-course of DH5 α (orange), DH5 α carrying 125 Kb plasmid pMB2 (grey) and 90 Kb plasmid pLMJ50 (yellow) in LB broth. The blue circles represent a no bacteria control.

terrific broth, Dubelcco's Modified Eagle Medium (DMEM, Invitrogen), Davis minimal medium and M9 minimal medium (data not shown). As a control, we used a 92 kb aggregative adherence *impAB* plasmid, pLMJ50³¹, which did demonstrate a significant carrying cost in DH5 α (Fig. 3).

Characterization of pMB2. To determine whether nutrient acquisition conferred by the pMB2 region harboring transport systems operons pMB2_00012/*hemR* and *sitABCD* located at 7,247–8,038 bp and 19,195–22,644 bp respectively on pMB2 offsets the carrying cost of pMB2, this segment of the plasmid was excised. A 32,331 bp segment that included these genes was deleted from the plasmid by excision with *NotI* (NEB), which cuts pMB2 at 122,777 bp, and *XbaI* (NEB), which cuts pMB2 at 29,449 bp. The restricted ends were filled in using *E. coli* DNA polymerase I (NEB) and then ligated on one other to generate a 93,450 bp autonomously-replicating

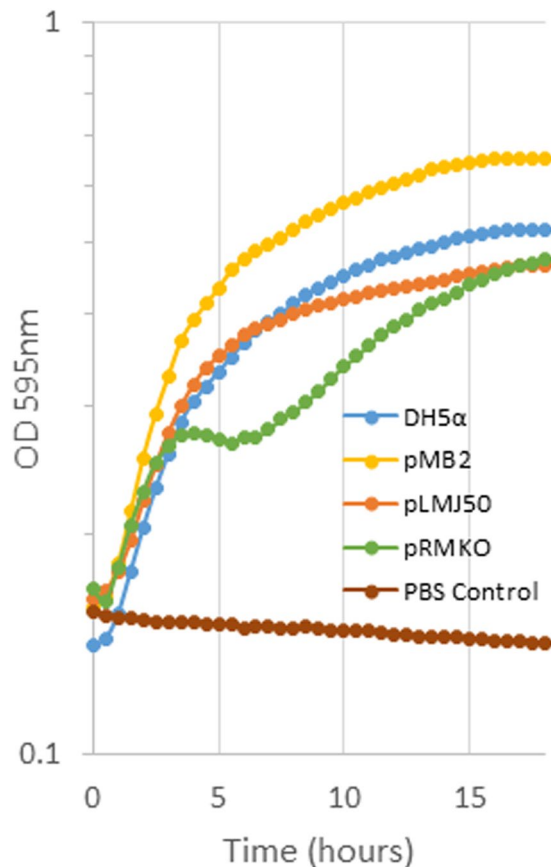


Figure 4. 18 h growth time-course of DH5 α carrying the pRMKO miniplasmid derived from pMB2 (green) compared to growth of DH5 α (blue), DH5 α carrying pMB2 (yellow) and 90 Kb control plasmid pLMJ50 (orange) in LB broth. Brown circles represent a no bacterial control.

mini-plasmid, pRMKO. The excised 32,331 bp fragment containing the transport system region was cloned into the *Xba*I and *Not*I sites of pBluescript II SK (Agilent) and named pRMC.

As seen in Fig. 4, the LB growth data, the equivalent or faster growth conferred by pMB2 was not seen with the mini-plasmid pRMKO, which grew at a rate that was slower than equivalent-sized plasmid pLMJ50. Intriguingly, pRMC, the cloned deleted region also conferred slower growth than wildtype, although in this case the carrying cost may be attributed to the higher copy number of the clone. Attempts to move the large insert to a lower copy-number vector were not successful and therefore it was not possible to fully complement the mini-plasmid deletion in *trans*. We therefore elected to functionally characterize the *sitABCD* genes that are within the deleted sequence.

We amplified the *sitABCD* operon and its promoter from plasmid pSIT1 (which has an identical copy of the genes) received from Laura Runyen-Janecky²⁶, using primers *sitF* and *sitR* and cloned them into pACYC177 to create pINK2301. In Davis minimal media iron-depleted by EDDA, DH5 α grows much slower than M63c, the *E. coli* strain from which pMB2 was isolated. Transformation of DH5 α with pMB2 produced a faster growth rate that was lower in DH5 α (pRMKO). As shown in Fig. 5, this growth defect could be complemented in *trans* with pINK2301.

To determine whether pMB2 enhanced bacterial fitness, we conducted competition experiments between strain M63c, the strain from which pMB2 was originally isolated, and commensal *E. coli* strain HS. We also competed DH5 α strains carrying pMB2 and pRMKO against one another. These experiments were performed in rich media (LB) as well as in DMEM in the absence of antibiotics, however antibiotics were used to select competitors on plates to perform counts. In both media, strains carrying pMB2 outcompeted commensal or laboratory strains. Conversely, DH5 α (pRMKO) suffered a competitive disadvantage against DH5 α (pMB2), as well as against plasmidless DH5 α (Fig. 6).

We sought to determine whether the plasmids were stably inherited and found that this was the case in source strain M63c over 200 generations. However, pMB2 and pRMKO were less stably inherited in a DH5 α background, with pRMKO proving to be less stably inherited than pMB2 in the medium term (Fig. 7).

Discussion

In this study, we identified and characterized pMB2 a multidrug resistance plasmid extracted from an *Escherichia* strain isolated in Nigeria. We were drawn to the plasmid by its *aac(6')-Ib-cr* gene but its resistance region also includes genes encoding resistance to seven other classes of antimicrobials, including the ESBL gene *bla_{CTX-M-15}*. There have been several reports of co-occurrence of *bla_{CTX-M-15}* and *aac(6')-Ib-cr* across of the globe, including in

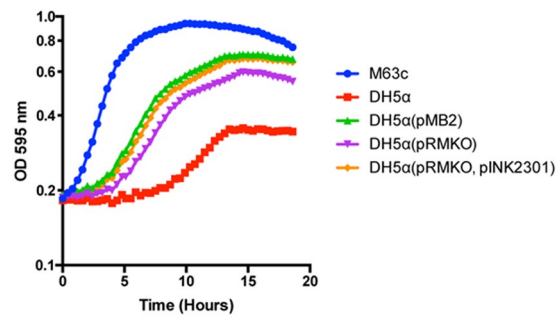


Figure 5. 18 h growth time-course in Davis minimal media pre-treated with deferrated EDDA to deplete iron. Wildtype M63c strain (grey) compared to DH5 α (orange), DH5 α carrying pMB2 (green), pRMKO miniplasmid (yellow), pRMKO miniplasmid plus the cloned *sit* genes in pINK2301 and 90 Kb control plasmid pLMJ50 (blue).

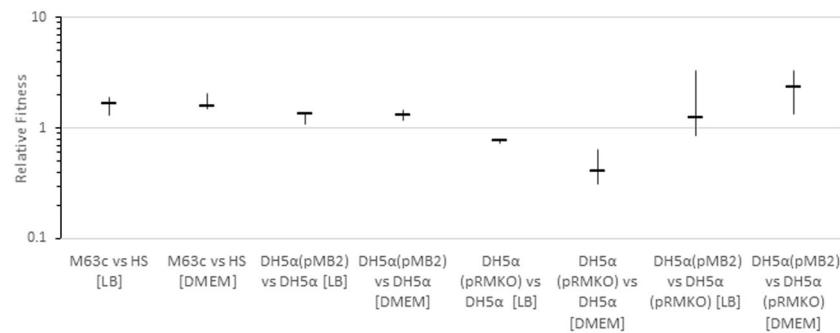


Figure 6. Relative fitness of the initially listed strain compared to the subsequently listed strain in rich media (LB) and minimal media (DMEM). Each data point is the mean of duplicate experiments and the error bars are the computed standard deviations.

Nigeria^{32–35}. This common co-resistance profile implies that ciprofloxacin use, which is growing¹, also selects for the carriage of ESBL genes and vice versa explaining why IncFII plasmids carrying these genes have traditionally been associated with successful pandemic clones^{36,37}. This report appears to be the first one directly confirming a genetic linkage of *bla*_{CTX-M-15} and *aac*(6′)-*Ib-cr* in Nigeria. However the structure of this plasmid in general and the resistance region in particular has been reported from round the world.

When resistance is conferred by a plasmid of considerable size, the associated metabolic burden on the organism due to the additional energy required in replication and gene expression typically exerts a carrying cost, at least theoretically³⁸. In practice, this is not always true but the co-evolutionary routes to reduced cost are often unknown. Some research has suggested that this burden is reduced or eliminated through compensatory mutations or acquisitions^{14,39–43}. Alternatively, or in addition, plasmid-encoded resistance genes themselves could confer a fitness advantage in the absence of antimicrobials^{14,41}.

Antimicrobial use is the principal driving force behind the current epidemic of antimicrobial resistance. However, research has shown that, once evolved, multidrug resistance plasmids can be maintained in the absence of selection⁴⁴. There is a dearth of fitness defects reported for resistance plasmids in the literature and the reasons why some of them are evolutionarily successful are not well understood^{41,45}. This study demonstrates that the *aac*(6′)-*Ib-cr* gene of strain M63c is carried on a large multidrug resistance plasmid pMB2, which also bears a number of genetic loci that support its evolutionary success irrespective of antibiotic selection. The 125 Kb plasmid does not have a carrying cost *in vitro* and competition experiments verified that 32 Kb region of the plasmid accounts for selective success. Included in this evolutionarily significant 32 Kb region are the *sitABCD* genes, which promote growth under iron-limited conditions, along with as yet unidentified loci that promote growth in rich and minimal media. The *sitABCD* and adjacent genes studied in our research are highly conserved among most of them and therefore the fitness they confer is also likely to be common. Overall, these data suggest that fitness conferring mobile elements associated with resistance can co-evolve with host bacteria to compensate the costs of their carriage and propagate its transmission irrespective of antibiotic selection. While *in vivo* fitness is yet to be evaluated for these loci, and should be an area of future study, the *in vitro* advantages conferred were substantial. Without a carrying cost large multidrug resistance plasmids could persist in commensal *E. coli* even in the absence of antimicrobial pressure. Future work on resistance plasmids and elements should focus more intently on fitness genes in addition to those that encode drug resistance.

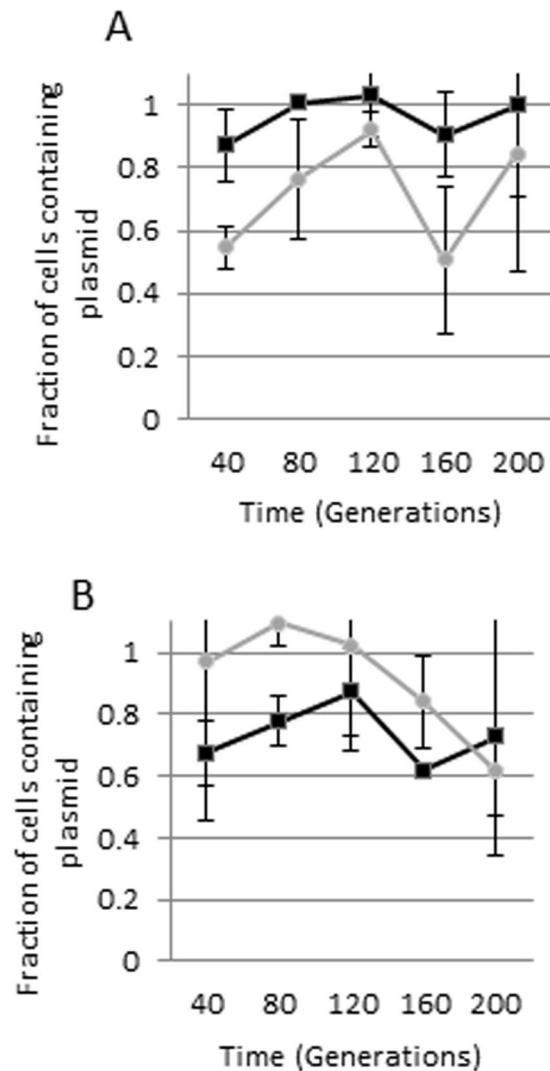


Figure 7. Plasmid stability in the absence of selection (A) stability of pMB2 in M63c, its natural host (dark line) and control plasmid pBR322 in DH5α (grey line) (B) stability of pMB2 (grey line) and pRMKO (dark line) in DH5α.

Methods

Ethical considerations. Permission to conduct this study was granted by the Ethics and Research committee of Obafemi Awolowo University Teaching Hospitals Complex, Ile-Ife with approval number IEC No. 00005422. Patients or their parents and guardians from whom the strains were isolated gave informed consent to participate in the study and all procedures were performed in accordance with the ethics committee's guidelines and requirements.

Strains. Strains used in this study are listed in Table 3. Commensal *E. coli* were collected from Nigerian mother-infant pairs where the infant suffered from diarrhea as previously described¹⁷. Altogether stool samples from 134 mothers-infant pairs, yielded 1,098 *E. coli* isolates for evaluation. Routine culture of bacteria was performed aerobically at 37 °C in Luria broth supplemented with chloramphenicol (30 µg/mL), ampicillin (100 µg/mL), tetracycline (25 µg/mL), or neomycin (50 µg/mL) where applicable. Strains were maintained at –70 °C in Luria broth:glycerol 1:1.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed using the Clinical and Laboratory Standards Institute (CLSI) disc diffusion method and interpreting zone diameters in accordance with CLSI guidelines in WHONET software version 5.3⁴⁶. Discs used contained ampicillin (10 µg/ml), streptomycin (10 µg), trimethoprim (5 µg), tetracycline (30 µg), nalidixic acid (30 µg), chloramphenicol (300 µg), sulphonamide (1000 µg) and ciprofloxacin (5 µg) (Oxoid/Remel). *E. coli* ATCC 35218 and DH5αE (Invitrogen) were used as control strains. Minimum inhibitory concentrations (MICs) for nalidixic acid and ciprofloxacin were determined using E-test (bioMérieux) on Mueller-Hinton (MH) agar. Kanamycin MICs were determined by agar dilution on Mueller-Hinton Agar.

	Genotype and description	Reference or Source
M63c	<i>E. coli</i> wt, S ^R , Nal ^R , Sul ^R , Amp ^R , Cip ^R , Te ^R , W ^R , K ^R	17
M63a, M63b, M63d, M63d	<i>E. coli</i> isolates recovered from the same specimen as M63c	17
C63a, C63c	<i>E. coli</i> isolate obtained from a specimen collected on the same day, from the infant of research participant whose stool yielded M63c	17
DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (rK ⁻ mK ⁺) <i>phoA supE44</i> λ - <i>thi-1 gyrA96 relA1</i>	Invitrogen
C600 Nal ^R	Nalidixic acid-resistant derivative of C600.	68
NCTC 10418	Susceptibility testing control	
ATCC 35218	Susceptibility testing control	
EC1502	Rifampicin-resistant, plasmid free <i>E. coli</i> strain	University of Bradford
HS	Commensal <i>E. coli</i> strain that has been shown to colonize non-virulently in human volunteer studies	69

Table 3. Strains used in this study.

General microbiology and molecular biology procedures. The Promega Wizard[®] kit was used for genomic DNA extractions and plasmids under 20 Kb were extracted using a Qiagen[®] MiniPrep Kit. Naturally-occurring low copy number larger plasmids were extracted, after growth of host bacterium in Terrific Broth and induction with chloramphenicol, by a modified boiling protocol for small scale preparations^{47,48} and using the Qiagen[®] Large Construct Kit for large-scale preparations. Table 4 lists the plasmids used in the study, their relevant properties, and their sizes. Plasmids were electroporated into *E. coli* host strains using a Bio-Rad micropulser according to manufacturer's instructions. Sequences of oligonucleotide primers used in the study are listed in Supplementary Table 1. Amplification cycles began with a two-minute hot start at 94 °C followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 5 °C below the melting temperature (unless otherwise specified) for 30 s, and extending at 72 °C for one minute per kilobase of DNA to be amplified. *Bst*CI restriction analysis of *aac*(6')-*Ib* PCR amplicons were used to determine whether they represented the ciprofloxacin resistance conferring *aac*(6')-*Ib-cr* allele as it only differs by two SNPs and is therefore indistinguishable by PCR⁴⁹. Up to two amplicons from each unique profile were sequenced to confirm PCR-RFLP-based classification. Where necessary, PCR amplicons were TA-cloned into the pGEMT vector (Promega) according to manufacturer's directions and plasmids were transformed into chemically competent *E. coli* K-12 TOP10 cells for sequencing. Other molecular biology operations were performed using standard procedures⁵⁰.

Bacterial identification. Bacteria were identified to the species level as described previously¹⁷ and further biotyped on the API20E system (bioMérieux). Genetic identification was performed by 16S ribosomal subunit sequencing, amplifying the gene encoding M63c's 16S rRNA with primers 10 and 1507R (Supplemental Table 1) in a thermocycler using the cycle at 94 °C for 2 m, [94 °C for 30 s, 59 °C for 45 s, 72 °C for 90 s] x36, and 72 °C for 10 m⁵¹. Multilocus sequence typing (MLST) was performed by amplifying the *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* genes using the primers of Wirth *et al.* as previously described^{52,53}. PCR amplicons for sequencing were size-verified by agarose electrophoresis, cloned into pGEMT, and electroporated into DH5 α E (Thermo Scientific) using a Biorad Micropulser. Plasmids were extracted with a Qiagen Plasmid Mini Kit and Sanger sequenced from M13F and M13R priming sites.

Shot-gun sequencing and sequence analysis. Whole-replicon shotgun library preparation, Sanger sequencing and assembly of plasmid pMB2 was performed by SeqWright DNA Technology Services (Houston, TX). Preliminary sequence analyses and annotation was performed manually by four authors in Artemis⁵⁴ with open reading frames initially predicted using Glimmer⁵⁵⁻⁵⁷ and basing gene annotations on 98% or greater identity at the nucleotide and amino acid levels if BLAST e-value was 0⁵⁸. Plasmid MLST typing was performed *in silico*, using PlasmidFinder and pMLST versions 2.0⁵⁹. Open Reading Frame (ORF) identity was determined using BLAST and Pfam^{56,57}. Two automated annotations were performed using BASys⁵⁵ and Prokka v 1.13.3⁶⁰. Five authors resolved annotation discrepancies among automated and manual annotations. Antimicrobial resistance genes and their genetic context were searched using Galileo AMR (<https://galileoamr.arcbio.com/mara/>)⁶¹ and ResFinder⁵⁸. Direct and inverted repeats were identified by dot-plot analysis of pairwise FASTA alignments made using the BLAST suite. ORF and feature plots were prepared using BLAST ring image generator⁶².

In vitro conjugation. *In vitro* conjugation experiments were performed by solid-surface matings. Donors and recipients were cultured in LB with appropriate selective antimicrobials. 0.5 mL of donor and recipient culture, grown overnight with selection, was spun down at 5000 rpm for 5 minutes in a fixed rotor microcentrifuge and resuspended in 20 μ l of LB without antibiotics. The suspension was spotted onto dried LB plates, allowed to dry at room temperature for 15 minutes and then incubated at 37 °C for three hours. The mating reaction was resuspended in 1 ml of LB with vortexing and placed on ice to terminate conjugation. After mating, serial ten-fold dilutions of each terminated reaction was made in cold phosphate buffered saline and plated onto plates containing tetracycline (or other appropriate antimicrobials for controls)—to select for the plasmid—and nalidixic acid—resistance to which is conferred chromosomally in the recipient. Transconjugant colonies were counted after overnight incubation at 37 °C and up to 50 colonies were verified by plasmid profiling, phenotype on MacConkey and Eosin methylene blue agars, PCR-RFLP for the *fliC* allele⁶³, and PCR for donor- and

Plasmid	Description	Size	Reference or source
pMB2	Naturally occurring <i>aac(6′)-Ib-cr</i> -bearing plasmid	125 Kb	This study and ¹⁹
pMB80-2	Naturally occurring conjugative plasmid from enteropathogenic <i>E. coli</i> strain	>100 Kb	⁴⁸
pRMKO	Mini-plasmid constructed by deleting a 32,331 bp <i>Not1 - Xba1</i> fragment from pMB2	93 Kb	This study
pRMC	pBluescript II SK containing a 32,331 bp <i>Not1 - Xba1</i> fragment from pMB2 cloned	35 Kb	This study
pBAD/Thio-TOPO	Arabinose inducible expression vector	4,454 bp	Invitrogen
pBluescript II SK +	High copy number cloning vector	2,961 bp	Agilent
pSIT1	Clone of the <i>Shigella sitABCD</i> genes		⁷⁰
pINK2301	<i>Shigella sitABCD</i> genes subcloned from pSIT1 into pACYC177	7.1 Kb	This study ¹⁹
pLMJ50	Cm ^R ; large aggregative adherence plasmid from EAEC strain 60A in which the <i>impB</i> gene is replaced with a <i>cat</i> cassette	90 Kb	³¹
pGEM-T	Amp ^R ; TA-cloning vector	3,000 bp	Promega
pBR322	Amp ^R , Tc ^R cloning vector	4,363 bp	NEB
pACYC177	Amp ^R , Km ^R cloning vector	3,941 bp	NEB

Table 4. Plasmids used in this study.

recipient-specific markers. Viable counts of donors and recipient were also performed. The number of transconjugant colonies per donor colony-forming units was computed as the plasmid transfer efficiency⁶⁴

Growth curves. Growth curves were plotted following growth of each strain in microtiter wells containing 160 µl of media in an Infinite[®] 2000 Pro Series microplate reader preset at 37 °C (Tecan). Commercially available medias were prepared as recommended by the manufacturers. Iron-depleted media was prepared by adding deferrated ethylene diamino-o-dihydroxyphenyl acetic acid (EDDA) as described previously⁶⁵. Test strains were first cultured overnight in 5 mL of LB media containing appropriate antibiotics where required. Overnight cultures were spun down, resuspended in 5 mL of 1X Phosphate Buffered Saline (PBS), and diluted to an optical density of 0.70 at 595 nm. 10 µl of the diluted cultures were then loaded into assigned wells in the microtiter reader. Absorbance was measured at 595 nm every 30 min preceded by 30 sec of orbital shaking for 24 hours. The average optical density of 25 reads per well at each time point was used in the analysis and up to six wells were tested per strain. Each specific strain and conditions was tested on a minimum of three occasions.

Plasmid stability. Plasmid stability was assessed by serially passaging *E. coli* strains carrying pMB2 and its derivatives as described by Sandegren *et al.*⁶⁶. Triplicate starting cultures were grown overnight at 37 °C in 1 mL of LB supplemented with ampicillin or tetracycline. Bacterial cells were washed, resuspended in LB without antibiotics and an aliquot of 100 µL of washed cells was inoculated into 10 mL of LB in a 50 mL conical tube and incubated overnight at 37 °C. 100 µL of this overnight culture was serially passaged to 1 mL of LB daily, approximating 10 generations of growth per passage. At select time points, samples were diluted and plated onto plain MacConkey plates and MacConkey plates containing tetracycline.

In vitro competition experiments. We measured relative fitness in traditional competition assays as outlined by Wiser and Lenski⁶⁷. Fitness was computed independently in LB broth and DMEM and *E. coli* strains carrying pMB2 and its derivative plasmids were selected using antibiotic markers present on those plasmids. Briefly, separate cultures of the competing strains were grown overnight in the test medium containing ampicillin at 37 °C before volumes equivalent to equal optical densities were added to a 50 mL conical tube to a final volume of ≈0.1 ml, which was immediately made up to 10 mL with test medium. The culture was mixed, 100 µL was withdrawn for viable counting (diluting with normal saline) and the culture was then incubated at 37 °C with shaking. At 24 h, 100 µL was withdrawn, diluted with normal saline, and plated out for counting. For wildtype strains, counting was performed on MacConkey agar plates containing ampicillin, tetracycline and no antibiotics. For strains carrying pMB2 or its mini-plasmid deletion construct, pRMKO, counting was performed on MacConkey agar plates containing ampicillin, Mueller Hinton tetracycline plates and Mueller Hinton trimethoprim plates. Differential computation of strains carrying pMB2 or pRMKO were made possible by the fact that pMB2's *dfrA17* gene, conferring trimethoprim resistance, is deleted from pRMKO. Fitness (*w*) was calculated by comparing the ratio of competed bacterial strains obtained at 24 h, *A_f* and *B_f* hours respectively, with those computed from the sample drawn at 0 h (*A_i* and *B_i*, respective), that is, using the formula specified by Wiser and Lenski⁶⁷

$$w = \frac{\ln(A_f/A_i)}{\ln(B_f/B_i)}$$

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Author contributions

I.N.O. conceived the study, which was designed by A.O.A. and I.N.O. R.M., M.B., O.C.-F., B.W.O., J.I.O.-V., N.K.Q. and I.N.O. performed experiments acquired the experimental data. R.M., M.B., O.C.-F., J.B., J.I.O.-V., L.P. and I.N.O. analyzed and interpreted the data. The initial draft was prepared by R.M., O.C.-F., A.O.A. and I.N.O. R.M., J.B., L.P., M.B. and I.N.O. prepared the figures. All the authors performed substantial revisions and approved the final version.

Competing interests

The authors declare no competing interests.

Additional information

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