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Original Research Article

Clinical antibiotic-resistance plasmids have small effects on biofilm formation and population growth in *Escherichia coli* in vitro

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ABSTRACT

Antimicrobial resistance (AR) mechanisms encoded on plasmids can affect other phenotypic traits in bacteria, including biofilm formation. These effects may be important contributors to the spread of AR and the evolutionary success of plasmids, but it is not yet clear how common such effects are for clinical plasmids/bacteria, and how they vary among different plasmids and host strains. Here, we used a combinatorial approach to test the effects of clinical AR plasmids on biofilm formation and population growth in clinical and laboratory *Escherichia coli* strains. In most of the 25 plasmid-bacterium combinations tested, we observed no significant change in biofilm formation upon plasmid introduction, contrary to the notion that plasmids frequently alter biofilm formation. In a few cases we detected altered biofilm formation, and these effects were specific to particular plasmid-bacterium combinations. By contrast, we found a relatively strong effect of a chromosomal streptomycin-resistance mutation (in *rpsL*) on biofilm formation. Further supporting weak and host-strain-dependent effects of clinical plasmids on bacterial phenotypes in the combinations we tested, we found growth costs associated with plasmid carriage (measured in the absence of antibiotics) were moderate and varied among bacterial strains. These findings suggest some key clinical resistance plasmids cause only mild phenotypic disruption to their host bacteria, which may contribute to the persistence of plasmids in the absence of antibiotics.

1. Introduction

Pathogens carrying antibiotic resistance (AR) plasmids are an increasing threat to public health, reducing treatment options for infected patients. But besides conferring resistance, AR plasmids can influence other phenotypic traits of their host bacteria, including biofilm formation (Burmølle et al., 2008; Dudley et al., 2006; Gallant et al., 2005; Gama et al., 2020; Ghigo, 2001; May and Okabe, 2008; Røder et al., 2013; Schaufler et al., 2016; Teodósio et al., 2012; Yang et al., 2008). This matters in the context of infectious disease because biofilms are often involved in persistent infections (Costerton et al., 1999; Lewis, 2001; Stalder et al., 2020). Therefore, in addition to conferring AR, any phenotypic effects of AR plasmids on biofilm formation may have downstream effects for bacterial epidemiological success and/or the likelihood of establishing persistent infections. More generally, plasmid-encoded changes in biofilm formation may influence evolutionary success, for both plasmids themselves and their host bacteria. In natural environments, bacteria very often live in biofilms, adhering to surfaces and embedded in an extracellular polymeric matrix (Flemming and Wuertz, 2019; Hall-Stoodley et al., 2004). This lifestyle can improve bacterial resistance to harsh environmental conditions (Lewis, 2001), and biofilms may be hotspots for plasmid transfer via conjugation (Burmølle et al., 2008; Ghigo, 2001; Røder et al., 2013; Virolle et al., 2020). Therefore, knowing whether and how plasmids affect bacterial biofilm formation would improve our fundamental understanding of the role of mobile genetic elements in bacterial evolution, and for clinically relevant AR plasmids this could increase our ability to predict and manage the spread of resistance in treatment contexts.

Despite both positive (Burmølle et al., 2008; Ghigo, 2001; Reisner et al., 2006; Stalder et al., 2020) and negative (Gallant et al., 2005) examples, there is no consensus on how AR plasmids influence biofilm formation. One possible reason for this is that most studies to date only considered one plasmid and/or one bacterial host strain (Gama et al., 2020; Lim et al., 2010; Røder et al., 2013; Teodósio et al., 2012). Given

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differences in strain construction, culture conditions, and other aspects of study design, this makes it hard to gain an overall view of how plasmids affect biofilm formation (i.e., to see the distribution of effect sizes). Therefore, individual studies testing multiple plasmids and strains simultaneously are required to estimate not only how plasmids affect biofilm formation, but how this varies among strains and plasmids. A second limitation of work to date is that many studies employed well-characterized laboratory plasmids and/or host strains (Burmølle et al., 2008; Gama et al., 2020; Ghigo, 2001). Clinical plasmids and strains may behave differently compared to laboratory-adapted versions, for example due to carriage of genetic elements, such as prophages or other plasmids, that can interfere with acquisition of new plasmids and their downstream phenotypic effects (Gama et al., 2020; Igler et al., 2022). A third aspect, which also helps to explain the lack of consensus over plasmid effects on biofilm formation, is that there are several possible physiological mechanisms involved (Barrios Gonzalez et al., 2005; Gama et al., 2020). The variables playing a role include the AR genes and accessory genes encoded on the plasmid, but also the chromosomal genes of the bacterial host (Holden et al., 2021). Teasing apart the contributions of these different drivers therefore requires 'swapping' plasmids across bacterial strains, testing each plasmid in multiple strains and vice versa, which is often challenging with clinical strains/plasmids.

Here, we aimed to measure the effects of clinical AR plasmids on biofilm formation in *E. coli*, and to do this for several different bacteria-plasmid combinations. To achieve this, we used a combinatorial approach, swapping plasmids across host strains and quantifying their effects on biofilm formation. This allowed us to distinguish effects caused by differences among strains, plasmids and both together. For clinical relevance, we included five AR plasmids isolated from patients and belonging to the most problematic resistance plasmid families, ESBL, KPC and OXA-48 plasmids (Carattoli, 2009; Poirel et al., 2018). As bacterial host strains, we included clinical *E. coli* isolates obtained from hospitalized patients, the native hosts of some of our plasmids, and well-studied laboratory *E. coli* strains that differ genomically at known loci. We quantified plasmid-mediated effects on biofilm formation in two different sets of experimental conditions, motivated by past work indicating that biofilm formation in clinical *E. coli* isolates can depend on growth conditions (Naves et al., 2008). Finally, we also tested for a possible link between altered biofilm formation and plasmid effects on bacterial population growth in the absence of antibiotics. Our rationale for this part was that past work showed plasmids have variable effects on bacterial growth rate (Alonso-del Valle et al., 2021; Baltrus, 2013; Rodríguez-Beltrán et al., 2021a, 2021b; San Millán and MacLean, 2019; Vial and Hommais, 2020), and the physiological drivers of these growth costs might also influence biofilm formation (Ricci et al., 2000). We found weak effects on biofilm formation for five clinical AR plasmids, specific to host background and nutrient condition. This provides new information about how AR plasmids influence biofilm formation and other phenotypic traits in epidemiologically successful, clinical bacteria.

2. Materials and methods

2.1. Plasmids

To test how natural, clinically relevant AR plasmids influence biofilm formation, we used five AR plasmids (pESBL1, pESBL15, pESBL25, pKPC-ecoli016 and a pOXA-48-like plasmid; Table 1). These were obtained from *E. coli* isolates from hospitalized patients in two different studies at the University Hospital Basel, Switzerland (Noll et al., 2018; Tschudin-Sutter et al., 2016). For simplicity, we refer to pKPC-ecoli016 (IncX3, IncU, 73.7 kb) and pOXA-48-like (IncL, 63.6 kb) as pKPC and pOXA-48, respectively, throughout the text. The plasmids pESBL1 (IncI, 111 kb), pESBL15 (IncI, 88.9 kb) and pESBL25 (IncFIA, IncFIB, 131 kb) encode extended spectrum β-lactamases (ESBL) of the CTX-M type. Plasmids pESBL1 and pESBL15 share a sequence similarity of 78% of coverage and 98.82% identity (Benz et al., 2020; Benz and Hall, 2023; Tschudin-Sutter et al., 2016). Plasmid pESBL25 was not previously described and shows a mosaic structure sharing similarity with other *E. coli* plasmids from the Genbank database (46%, 69% coverage and 99.77%, 99.81% identity with CP088793.1 and CP069982.1, respectively). The resistance genes encoded by pKPC and pOXA-48 belong to the carbapenemases, a subgroup of β-lactamases. pKPC variants were previously found in *Klebsiella pneumoniae* (100% coverage and 99.42%, 99.86% identity with CP027700.1 and MF150120.1, respectively). The pOXA-48-like plasmid shares a 97% coverage and > 99% identity both with the first described pOXA-48 plasmid (Poirel et al., 2012) and with pOXA-48_K8, one of the best studied pOXA-48-like variants (León-Sampedro et al., 2021). All five plasmids used here confer resistance to ampicillin (MIC >512 mg/L).

2.2. Bacteria and growth conditions

We used five *E. coli* strains (two clinical isolates and three laboratory strains) to investigate bacterial host-specificity of plasmid-mediated biofilm effects (Table 2). We included two clinical *E. coli* isolates, cESBL1 and cESBL15, cured of their native ESBL plasmids, originating from the clinical donor strains of the plasmids pESBL1 and pESBL15 (Benz et al., 2020). The antibiotic resistance profiles of these isolates are described in Benz et al. (2020), where cESBL1 corresponds to D1 and cESBL15 to D4, with the difference that the cured isolates used here are susceptible to ampicillin due to the loss of their native AR plasmids. The clinical isolate cESBL1 belongs to the *E. coli* phylogroup G (sequencing type (ST) 117), which has been associated with poultry and various mammals as well as severe extra-intestinal diseases in humans and is characterized by high virulence and enhanced antibiotic resistance potential (Clermont et al., 2019; Lu et al., 2016). The second clinical isolate, cESBL15, is part of the B1 phylogroup in *E. coli* (ST40), a group adapted to a broad spectrum of hosts but predominantly found in vertebrate animals including humans and frequently harbouring extended-spectrum β-lactamases (Bajaj et al., 2016; Berthe et al., 2013; Clermont et al., 2013; Martak et al., 2020). In addition, we used three isogenic laboratory strains, *E. coli* K-12 MG1655 (wild-type, hereafter referred to as MG1655), a variant thereof encoding a chloramphenicol resistance gene (*cat*) inserted into the *galK* gene locus (Δ*galK::cat*) and a chromosomal dTomato marker (hereafter referred to as MG1655 + Cm), and another variant carrying a K43R mutation in the ribosomal *rpsL* gene, providing resistance to streptomycin (hereafter referred to as MG1655 + Stm). We cultivated bacteria in lysogeny broth (LB) or in M9 minimal medium supplemented with 0.8% glucose and 1 mM $MgSO_4$. Overnight cultures prior to the experiments were supplemented with 100 mg/L of ampicillin to prevent plasmid loss, while no antibiotics were added during the biofilm formation assay.

Table 1
Resistance plasmids.

Plasmid	Incompatibility group	Size (kb)	Resistance genes	Reference (Genbank Ac. No.)
pESBL1	IncI	111	*aadA5*, $bla_{CTX\text{-}M\text{-}1}$, *sul2*, *dfrA*	SAMN17073775
pESBL15	IncI	88.9	$bla_{CTX\text{-}M\text{-}1}$	SAMN12275742
pESBL25	IncFIB, IncFIA	131	$bla_{CTX\text{-}M\text{-}14}$, *tetB*, *dfrA*, *mphA*	SAMN17073781
pKPC	IncX3, IncU	73.7	bla_{KPC2}, *sat2A*	UWWZ01000004.1
pOXA-48	IncL	63.6	$bla_{OXA\text{-}48}$	UWXP01000003.1

Table 2
Bacterial host strains.

Strain	Species	Sequence type	Plasmids	Notes	References
cESBL1	*E. coli*	117	IncF, ColRNAI	Clinical isolate, after curing pESBL1	Benz et al., 2020; Tschudin-Sutter et al., 2016
cESBL15	*E. coli*	40	ColRNAI, Col156	Clinical isolate, after curing pESBL15	Benz et al., 2020; Tschudin-Sutter et al., 2016
K-12 MG1655	*E. coli*	10	–	Laboratory strain, wild type	Blattner et al., 1997
MG1655 + Cm	*E. coli*	10	–	Laboratory strain, Cm^R, Δ*galK::cat*	Baumgartner et al., 2020
MG1655 + Stm	*E. coli*	10	–	Laboratory strain, Stm^R, *rpsL* K43R	Baumgartner et al., 2020
ATCC 14028	*S. enterica* Typhimurium	19	–	Cm^R, *marT::cat*	Diard et al., 2017

2.3. Construction of novel plasmid-host combinations by conjugation

We introduced each of the five plasmids to each of the five bacterial host strains by conjugation (Alonso-del Valle et al., 2021), resulting in 25 different transconjugants. Briefly, we diluted overnight cultures (LB) of recipient and donor strains 1:100 and incubated them for 3.5 h at 37 °C. In late exponential growth phase, we harvested cells by centrifugation (1500 *g*; 15 min) and resuspended them in 0.9% NaCl, then mixed the recipient and donor cultures in a 1:1 (*v*:*v*) ratio. Subsequently, we plated serial dilutions on selective agar plates. For the two strains MG1655 + Cm and MG1655 + Stm, we obtained transconjugants by using selective plates containing ampicillin (100 mg/L) and chloramphenicol (25 mg/L) or streptomycin (100 mg/L), respectively. To obtain transconjugants of the wild-type MG1655 strain and the cured clinical donor strains, cESBL1 and cESBL15, we used a *Salmonella enterica* Typhimurium strain ATCC 14028 harbouring a chloramphenicol resistance marker (*marT::cat*) (Diard et al., 2017) as intermediate plasmid host, allowing us to discriminate *E. coli* transconjugants from *S. enterica* transconjugants on chromatic agar plates containing ampicillin (100 mg/L), because these three host strains do not carry any chromosomal resistance markers. We confirmed plasmid carriage and strain ID (for cESBL1 and cESBL15 transconjugants) by colony PCR. For colony PCRs, we first obtained template DNA by touching single colonies with a sterile pipette tip and suspending the cells in 20 µL nuclease-free water. Then, we boiled the DNA at 95 °C for 15 min and added 2.5 µL to 22.5 µL PCR reagents. The PCR reaction mix contained 2× GoTaq® G2 HS Green master mix and 1 µM of the respective forward and reverse primer (see Table S1). Using a labcycler (Sensoquest, Göttingen, Germany), we ran the following thermal cycle program: initial denaturation for 4 min at 95 °C followed by 25 cycles of denaturation (30s at 95 °C), annealing (45 s at 65 °C) and extension (1 min at 72 °C) and final extension at 72 °C for 5 min.

2.4. Biofilm formation assays

We measured plasmid-mediated changes in biofilm formation for each plasmid-host combination in two sets of conditions: in LB medium and in M9 minimal medium. We performed an adapted version of the crystal violet assay described by Christensen et al., 1985 and O'Toole et al., 1999. Briefly, we inoculated three single colonies of each strain in randomly assigned wells containing 200 µL medium (LB or M9) of three separate 96-well microplates, resulting in 9 biological replicates of each of the 30 strains used (5 plasmids in each of the 5 hosts, plus 5 plasmid-free hosts). After overnight incubation at 37 °C with agitation, using a 96-pin replicator, we inoculated test plates containing 180 µL per well of the respective medium from the overnight cultures (1:180 dilution). After 24 h static incubation at 37 °C (in a plastic bag to minimise evaporation), we measured optical density at 600 nm using a Nano-Quant Infinite M200 Pro plate reader. Subsequently, we discarded the medium containing non-attached cells and washed the plates three times with 0.9% NaCl before staining with 0.1% crystal violet for 15 min. Then, we repeated the three washing steps and air-dried the plates for 1 h. We dissolved the retained crystal violet in 96% ethanol and measured absorbance at 595 nm. We performed the biofilm assays in LB and M9 minimal media in separate experiments.

2.5. Calculation of biofilm formation scores

We calculated three different measures of biofilm formation. First, "absolute biofilm formation" describes the amount of cells adhering to the polystyrene plate wells and is measured by absorbance after staining with crystal violet and re-dissolving in ethanol (OD595nm CV). Second, "normalised biofilm formation" gives the density of adhering cells relative to those in the liquid phase, calculated by dividing absolute biofilm formation (OD595nm CV) by the respective culture absorbance value (OD600nm culture). This normalisation step accounts for any potential effects of variability of final culture density before staining. Third, to quantify the change in biofilm formation caused by the presence of the plasmid in a given strain-plasmid combination, we calculated "relative biofilm formation" based on the normalised biofilm scores using the following formula:

$$Rel.\ biofilm\ formation = \frac{\left(\frac{OD595nm_{CV}}{OD600nm_{culture}}\right)_{transconjugant}}{\left(\frac{OD595nm_{CV}}{OD600nm_{culture}}\right)_{plasmid-free\ host}}$$

where values for the transconjugant come from a single microplate well (one replicate), and values for the plasmid-free host are the median for the corresponding plasmid-free strain (all replicates within a given plasmid-free host strain, because in our experimental design transconjugant replicates were not paired with individual plasmid-free replicates). This allowed us to obtain relative biofilm scores for each transconjugant replicate culture. For completeness, we also display the non-normalised biofilm formation scores in Fig. S1, which shows the relative biofilm formation values obtained by directly dividing OD595nm CV of the transconjugants by OD595nm CV of the plasmid-free strains. We calculated the error associated with individual biofilm formation estimates using the following error propagation formula (Silva et al., 2011):

$$\frac{\delta Q}{|Q|} = \sqrt{\left(\frac{\delta a}{a}\right)^2 + \left(\frac{\delta b}{b}\right)^2 + \ldots + \left(\frac{\delta z}{z}\right)^2}$$

a, b, …, z = measurement values; δ = error associated with measurement value; Q = calculated value from measurements a, b, …, z (in this study for example relative biofilm formation).

2.6. Growth curves and plasmid effects on bacterial growth

We determined plasmid growth effects in all host strains in liquid LB medium and M9 minimal medium by monitoring optical density in the absence of antibiotics in microplates over 24 h (see Fig. S2), then comparing transconjugants with their corresponding plasmid-free hosts. From a subset of the overnight cultures used for the biofilm formation assay above, we diluted three biological replicates of each strain into fresh medium using a pin replicator and incubated for 24 h at 37 °C in a plate reader (Tecan NanoQuant Infinite M200 Pro). We measured absorbance at 600 nm every 15 min after 5 s of shaking (amplitude = 1.5 mm). We determined the maximum growth rate (µmax), maximum optical density (OD_{max}) and area under the curve (AUC) using the growthrates (Petzoldt, 2017) and flux (Jurasinski et al., 2022) packages in R, version 0.8.2. Further, we calculated the relative maximum growth

rate by dividing the maximum growth rate of each transconjugant by the maximum growth rate of the respective plasmid-free host (as for relative biofilm formation). In this case, due to normally distributed data, we used the arithmetic mean. We calculated the errors using error propagation as above.

2.7. Statistical analysis

We tested for variation of biofilm formation (relative, normalised or absolute; see above) among strains and plasmids using a two-way ANOVA. We tested the significance of individual effects (the difference between plasmid-carrying and plasmid-free versions in each strain-plasmid combination) by Welch's two-sample *t*-test using the Holm-Bonferroni correction for multiple testing (Dunn, 1961; Holm, 1979; Neyman and Pearson, 1928). To estimate effect sizes as percentage changes in biofilm formation relative to plasmid-free strains, we took the average relative biofilm formation score in each combination, converted it to a percentage change (subtracting 1 then multiplying by 100), before removing negative signs (by squaring and rooting each value) to give all values as a percentage deviation in either direction.

3. Results

3.1. Weak effects of clinical antibiotic resistance plasmids on biofilm formation

Across the 25 combinations of five clinical plasmids (belonging to four different incompatibility groups) and five bacterial strains we tested in nutrient-rich LB medium (Fig. S3), changes in biofilm formation caused by plasmid introduction were on average weak (Fig. 1A; mean relative biofilm formation = 1.14, s.d. = 0.46). This amounted to an average effect size of ±18.61%, s.d. = 39.31 (in either direction, calculated from the squared and rooted mean percentage change in each combination). Despite this weak average effect across the dataset, there were some relatively strong individual effects (Fig. 1B), with a magnitude that varied depending on both the plasmid (two-way ANOVA: $F_{4,200} = 40.67$, $p < 0.001$), the host strain ($F_{4,200} = 19.15$, $p < 0.001$), and their interaction ($F_{16,200} = 29.76$, $p < 0.001$). Specifically, there were only three plasmid-host combinations where we detected significant changes in biofilm formation upon plasmid introduction (tested by Welch's two-sample *t*-test with Holm-Bonferroni correction, Fig. 1B). The strongest such effect was an increase by 214% in biofilm formation induced by plasmid pESBL25 in the clinical isolate cESBL15 (Fig. 1B). This combination was strikingly different from our observations in other combinations, so we carried out an additional experiment using six independently constructed clones of this plasmid-host combination. This revealed no such increase in biofilm formation (Fig. S5), suggesting the outlying measurement in the main dataset was specific to that particular assay and, therefore, this individual observation should be interpreted with caution. If we exclude this combination from the above analyses, the qualitative outcome is unchanged (effects of host strain, plasmid and interaction remain significant at $p < 0.001$), although the average effect size drops to ±11.06%, s.d. = 11.17. The other two significant changes ($p < 0.05$) we detected upon plasmid introduction were both in strain MG1655 + Stm, with plasmids pKPC and pOXA-48 (Fig. 1). Although we also observed high absolute biofilm formation in MG1655 + Stm upon acquisition of pESBL15 (Fig. S6), this change was not significant ($p > 0.05$). In summary, plasmids did not significantly alter biofilm formation in most combinations, and in the few cases where they did, these effects were specific to particular plasmid-host combinations.

We found further evidence that host genetic background matters for biofilm formation by comparing the five plasmid-free host strains: normalised biofilm formation scores of the plasmid-free strains varied significantly (one-way ANOVA: $F_{4,40} = 55.40$, $p < 0.001$; Fig. S3), with MG1655 + Stm scoring highest. MG1655 + Stm carries a K43R mutation in the ribosomal *rpsL* gene, but is to the best of our knowledge otherwise isogenic to the wild-type MG1655 strain. The comparison between the plasmid-free MG1655 and MG1655 + Stm strains therefore suggests the mutation in the *rpsL* gene promoted biofilm formation in plasmid-free *E. coli*. Furthermore, plasmid-carrying versions of this bacterial strain consistently had relatively high absolute and normalised biofilm scores (Fig. S3, S4, S6). This shows the effect of the *rpsL* mutation on biofilm formation was maintained after plasmid acquisition.

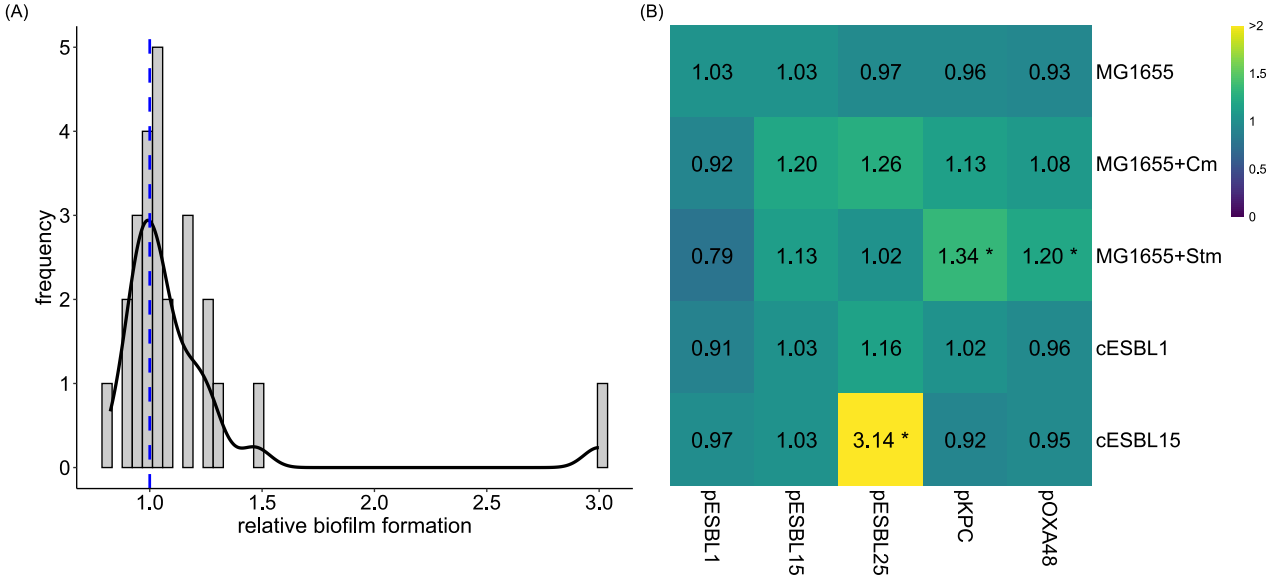

Fig. 1. A: Distribution of relative biofilm-formation scores (plasmid-carrying vs plasmid-free) for 25 different bacterium-plasmid combinations in LB medium. The score for each combination is taken as the median from nine replicate assays (see Methods); a score of 1.0 indicates no change in biofilm formation upon plasmid introduction. Frequency equals the number of cases with a given score. B: Variation of relative biofilm formation scores among the five host strains (columns) and five plasmids (rows); as in the left-hand panel, each score is the median from nine replicates (normalised and absolute scores for all replicates are provided in Fig. S3 and S6). Asterisks indicate combinations with $p < 0.05$ (Welch's two-sample t-test comparing plasmid-carrying vs plasmid-free, corrected for multiple testing using the Holm-Bonferroni method). Note the colour scale does not cover the whole range of data.

3.2. Similar weak effects of plasmids on biofilm formation in minimal growth medium

We tested whether our results were specific to LB medium by carrying out a separate biofilm assay in M9 minimal medium. This revealed a similar pattern to that observed in LB medium (Fig. 2): on average, plasmid introduction did not lead to large differences in biofilm formation compared with corresponding plasmid-free strains (Fig. 2A; mean relative biofilm formation = 1.29 ± 1.38, mean effect size =37.67%, s.d = 135.74). We detected a significant individual effect only for the same combination that also had the largest biofilm effect in LB (cESBL15 with pESBL25; Fig. 2B). Repeating the analysis without this combination, the average effect size decreased to ±10.57%, s.d. = 8.01, and there was no significant variation of average effects among plasmids (two-way ANOVA: $F_{4,192} = 0.40$, $p > 0.05$), although as in LB there was variation among host strains (two-way ANOVA: $F_{4,192} = 9.61$, $p < 0.001$) and the strain × plasmid interaction (two-way ANOVA: $F_{15,192} = 2.16$, $p < 0.01$). As in LB, strain MG1655 + Stm was the strongest biofilm former among plasmid-free strains (one-way ANOVA on normalised biofilm formation scores: $F_{4,40} = 129.50$, $p < 0.001$; Fig. S4), and remained so after plasmid acquisition. In summary, the experiment in minimal medium was consistent with our findings in rich medium, showing that plasmids had only moderate effects on biofilm formation in most combinations, and supporting the effect of the *rpsL* mutation carried by MG1655 + Stm on biofilm formation.

3.3. Moderate host-dependent effects of plasmids on bacterial growth rate

We measured population growth rates in the absence of antibiotics for all 25 plasmid-host combinations and their plasmid-free equivalents (Fig. S2). This revealed moderate negative effects of plasmids on bacterial growth rate on average: mean relative maximum growth rate after plasmid acquisition across all plasmid-host combinations was 0.94, s.d. = 0.08 in LB medium and 0.91, s.d. = 0.11 in M9 minimal medium (Fig. 3). We next tested for variation of average growth effects across strains and plasmids. This showed the five host strains paid different average growth costs upon plasmid acquisition (effect of strain in two-way ANOVA: $F_{4,50} = 3.08$, $p < 0.05$), although the strength of this effect depended on the plasmid (strain × plasmid interaction: $F_{16,50} = 2.55$, $p < 0.01$). For example, strain MG1655 paid relatively large costs for some plasmids, such as pOXA-48, but not for pESBL25 in LB medium (Fig. 3A). Despite this, different plasmids were not associated with different growth costs on average ($F_{4,50} = 1.34$, $p > 0.05$). In M9 minimal medium there was also variation among host strains ($F_{4,50} = 6.78$, $p < 0.001$) and plasmids ($F_{4,50} = 2.58$, $p < 0.05$), but no strain × plasmid interaction ($F_{16,50} = 1.44$, $p > 0.05$). The plasmid-bacterium combination cESBL15 with pESBL25 paid the largest growth cost in LB and in M9 (Fig. 3). This was the same combination where we observed the highest biofilm formation scores, which were not reproducible in other clones (Fig. S5). In most strain-plasmid combinations, individual effects were non-significant based on pairwise testing adjusted for multiple comparisons (Fig. 3). We note pairwise comparisons here have limited power to detect weak significant effects in individual cases, because of the smaller sample size within combinations compared to the biofilm dataset above ($n = 3$ here instead of 9 as for biofilm formation). From the same dataset, we extracted other growth parameters: maximum population density (maximum OD) and the area under the curve (AUC). These parameters provide a similar picture compared to growth rate, with moderate effects that varied among host strains (Fig. S7, S8). Finally, we tested for an overall association between growth costs and biofilm formation across the 25 strain-plasmid combinations, but found no evidence of this (Fig. S9). In summary, changes to bacterial growth rate in the absence of antibiotics upon plasmid acquisition were mostly weak, but depended on the bacterial host strain.

4. Discussion

In a combinatorial screen, we aimed to quantify plasmid effects on biofilm formation and growth across different strain-plasmid combinations, zooming out from individual strains, and to do this in two different nutrient conditions in vitro. Thus, we used a fully factorial design with clinical AR plasmids and a combination of clinical and laboratory host strains. In summary, the distribution of plasmid effects in our dataset revealed changes in biofilm formation that were in general moderate: clinical plasmids did not significantly alter biofilm formation in most combinations, and when they did their effects were weak. Furthermore, our experimental design allowed us to disentangle the contributions of plasmid and host strain to the biofilm formation. This showed both

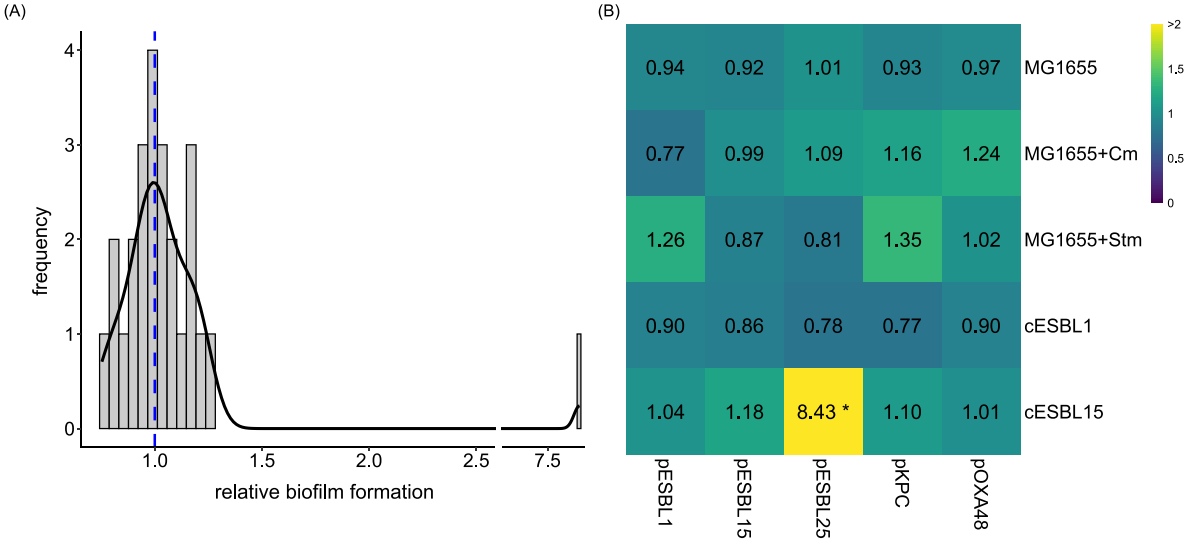

Fig. 2. A: Distribution of relative biofilm-formation scores (plasmid-carrying vs plasmid-free) for 25 different bacterium-plasmid combinations in M9 minimal medium. The score for each combination is taken as the median from nine replicate assays (see Methods); a score of 1.0 indicates no change in biofilm formation upon plasmid introduction. Frequency equals the number of cases with a given score. B: Variation of relative biofilm formation scores among the five host strains (columns) and five plasmids (rows); as in the left-hand panel, each score is the median from nine replicates (normalised and absolute scores for all replicates are provided in Fig. S4 and S6). Asterisks indicate combinations with $p < 0.05$ (Welch's two-sample *t*-test comparing plasmid-carrying vs plasmid-free, corrected for multiple testing using the Holm-Bonferroni method). Note the colour scale does not cover the whole range of data.

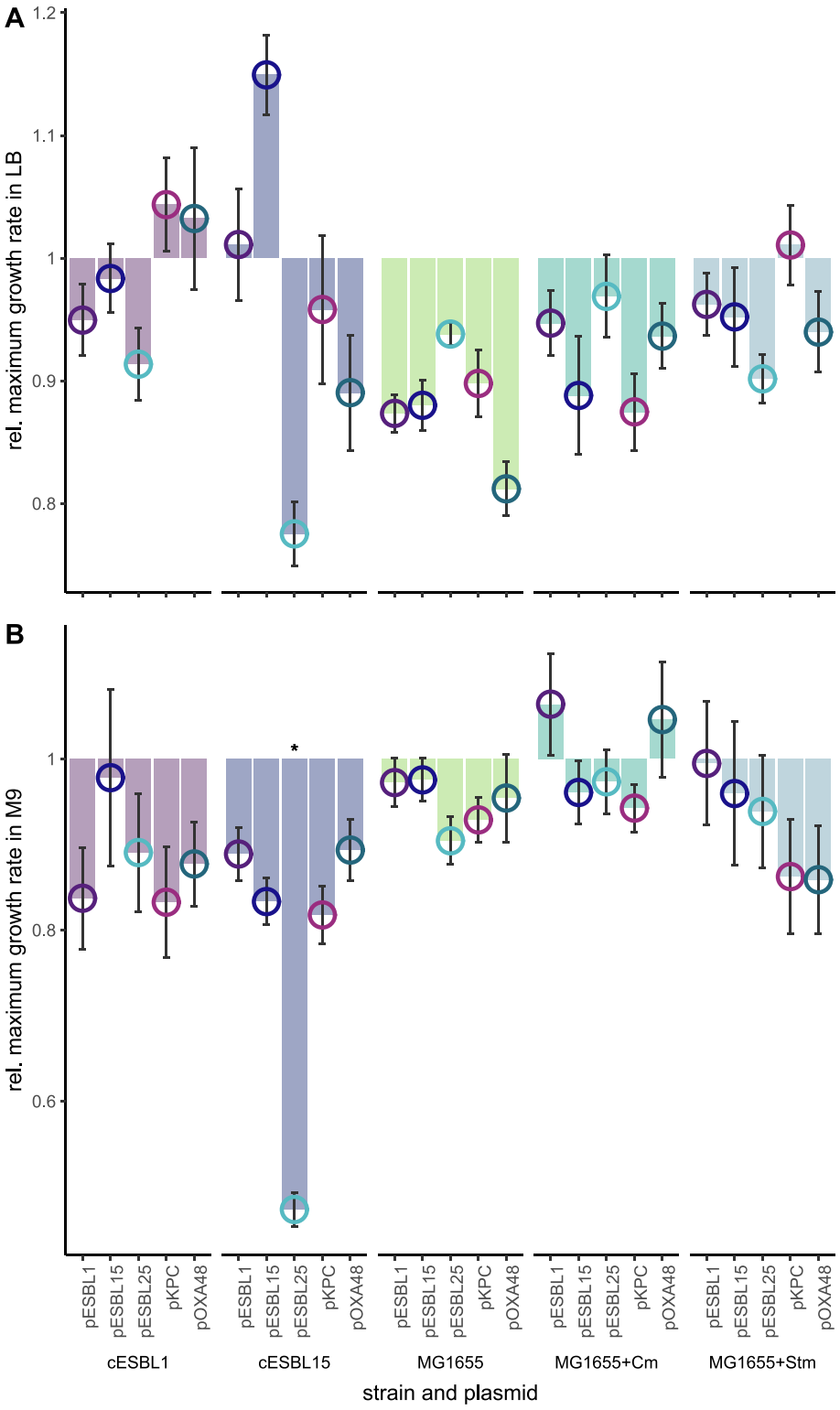

Fig. 3. Relative growth rates (of plasmid-carrying strains relative to plasmid-free equivalents) in all combinations in LB medium (A) and M9 minimal medium (B). Cases with $p < 0.05$ (Welch's two sample *t*-test comparing plasmid-carrying vs plasmid-free, corrected for multiple testing using the Holm-Bonferroni method) are indicated with an asterisk ($n = 3$). Error bars indicate propagated standard error.

factors can influence biofilm formation, with the host strain having a relatively strong influence. A key implication of this is that plasmid effects on bacterial ecology can be specific to the plasmid-host combination. Thus, predicting the spread of plasmid-encoded resistance genes and their downstream effects on biofilm formation requires information not only about which plasmids are circulating, but also which strains they are carried by.

Our results contrast with several past observations of plasmids affecting biofilm formation (Burmølle et al., 2008; Gallant et al., 2005; Ghigo, 2001; Reisner et al., 2006; Schaufler et al., 2016; Yang et al., 2008). One possible reason for this, which could potentially be explored in future work, is the role of mating-pair formation and conjugation itself. Some past studies showed strong effects of plasmids on biofilm formation upon co-inoculation with viable plasmid recipient strains (Ghigo, 2001; Reisner et al., 2006; May and Okabe, 2008). Ghigo (2001) proposed a key role for pilus expression, followed by conjugation and transient derepression of pilus synthesis in new transconjugant cells. In our experimental set-up, with pure cultures of plasmid-carrying strains, there are no/fewer recipient cells than in such co-inoculation experiments, and transfer to other plasmid-carrying cells is probably limited by surface exclusion (Achtman and Kennedy, 2023). Consistent with this, we identified putative entry exclusion system genes for all the plasmids tested here (Table S2). The biofilm effects we observed are therefore more likely to stem from the plasmid's interaction with the bacterial host's gene expression and metabolism (Barrios Gonzalez et al., 2005; Gama et al., 2020). Nevertheless, we do not rule out that these plasmids would have stronger effects in other conditions where conjugative transfer is more common, particularly because these plasmids are conjugative (Benz et al., 2020; Pathak et al., 2022; Teodósio et al., 2012). Ghigo (2001) also observed plasmid effects on biofilm formation for several plasmids in pure cultures, but using a different method to quantify biofilm formation, namely on pyrex slides in continuous culture, whereas we measured biofilm formation in static cultures on a plastic surface. This suggests other factors contribute to the strength of observed biofilm effects, besides the difference between pure and mixed cultures. Consistent with this, some other past studies used pure-culture set-ups that are more similar to ours and still found relatively strong biofilm effects (Burmølle et al., 2008; Gallant et al., 2005; Schaufler et al., 2016).

In contrast to the mild biofilm effects we observed upon plasmid acquisition, we found a strong effect of a chromosomal resistance mutation (*rpsL* K43R in *E. coli* K-12 MG1655), resulting in a significantly higher baseline biofilm formation which was maintained after plasmid acquisition. This effect is consistent with previous studies linking *rpsL* mutations to pleiotropic changes in other bacterial phenotypes (Paulander et al., 2009) and biofilm formation in particular (Boehm et al., 2009). More generally, chromosomal resistance mechanisms frequently affect essential and/or conserved genes, such as ribosomal genes like *rpsL* and those encoding RNA polymerase subunits, often leading to wide-ranging effects on expression of other genes and phenotypes (Hall et al., 2015; Perkins and Nicholson, 2008; Qi et al., 2014). The relatively strong side effects we observed for a chromosomal resistance mutation compared to plasmid-borne resistance may therefore be consistent with a wider trend that plasmid-borne resistance comes with relatively few pleiotropic effects compared to, for example, resistance mutations affecting ribosomal subunits or RNA polymerase (Rodríguez-Beltrán et al., 2021a, 2021b). We speculate further that relatively mild effects of clinical resistance plasmids on both biofilm formation and population growth may contribute to their stability in the absence of antibiotics, in that bacteria can acquire them without incurring large costs. This is also consistent with past work finding a similarly low cost of plasmid-borne resistance (Vogwill and MacLean, 2015; see also Alonso-del Valle et al., 2021; we discuss growth cost in more detail below). Note this does not exclude the idea that some plasmids which increase biofilm formation may gain an evolutionary advantage, due for example to increased opportunities for horizontal transfer and improved survival of their bacterial hosts (Burmølle et al., 2008; Ghigo, 2001; Røder et al., 2013). Plasmids may be under positive selection for such effects in some conditions, but our experiments suggest strong changes in biofilm formation are not general, in that they did not arise in most of the plasmid-bacterium combinations we tested in vitro. Because we observed similar biofilm effects in the two clinical isolates (with the exception of one specific plasmid-host combination) and the three laboratory host strains, we suggest the moderate plasmid effects we detected are likely to apply in other strains as well. The inclusion of clinical strains is important here, and will be in future work expanding the range of strain-plasmid combinations tested, because they can differ from model strains by carriage of additional genetic elements such as plasmids, prophages and pathogenicity islands, which can potentially interfere with the phenotypic effects of new plasmids (Gama et al., 2020; Igler et al., 2022).

Consistent with the moderate phenotypic effects in our biofilm dataset, plasmids also had only moderate effects on bacterial growth in the absence of antibiotics. Overall, this agrees with the weak growth effects of a clinical pOXA-48 plasmid in natural enterobacterial strains reported by Alonso-del Valle et al. (2021). It also aligns with the results of a meta-analysis by Vogwill and MacLean (2015). In their analysis of 77 publications, Vogwill and MacLean (2015) found an average relative growth of 0.91 ± 0.024 for AR plasmids, across studies employing different methodologies and fitness measures. The average relative growth rate in our dataset of 0.94 ± 0.08 in LB and 0.91 ± 0.11 in M9 minimal Medium is comparable to this. By testing several strain-plasmid combinations, we also found the bacterial host genotype to be a key driver of both average plasmid growth effects and the observed differences among plasmids. For example, plasmid pESBL15 conferred a positive growth effect in host strain cESBL15 in LB (Fig. 3A), but was relatively costly in other strains (Fig. 3A). Similarly, in Mminimal Medium, pOXA48 conferred a higher growth cost in strains cESBL1, cESBL15 and MG1655 + Stm compared to the other strains (Fig. 3B). Furthermore, our data suggest even small genetic differences between strains can significantly alter the growth costs associated with plasmids, shown for example by the relatively high cost of pKPC in strains MG1655 and MG1655 + Cm compared to strain MG1655 + Stm in LB (Fig. 3A). Across the two growth conditions we tested, the overall picture was similar, although some individual effects differed between LB and M9 minimal medium, such as the effect of pESBL15 in strain cESBL15. Note our power to test the significance of individual pairwise effects here is limited due to the relatively small samples size within combinations, and the need to adjust for multiple comparisons. Thus, our findings support the moderate average effects, and variability among different strains, strain-plasmid combinations and environmental conditions, of plasmid growth effects observed in previous studies (Alonso-del Valle et al., 2021; Vogwill and MacLean, 2015; Fernandez-Calvet et al., 2023), and show how this applies across various combinations including relevant clinical strains/plasmids.

Even though we were able to visualise the distribution of plasmid effects on biofilm formation across 25 combinations in two different experimental conditions, our dataset has certain limitations. First, the number of significant effects in our dataset was small. In a larger screen with even more combinations, a clearer picture could be obtained of the variance and mean effect among the subset of combinations where plasmids do alter biofilm formation and/or growth rate. We note there is a significant practical hurdle to scaling up the combinatorial approach used here, in terms of plasmid curing and re-introduction effort increasing as more strains are added. Second, one strong biofilm effect (combination cESBL15 with pESBL25) was not reproducible in further validation experiments (Fig. S5), and we suggest to consider the results for both biofilm formation and growth costs for this particular combination with caution. This emphasizes that individual observations should be interpreted carefully, especially when using only one transconjugant clone per combination (an approach we adopted in our initial combinatorial screen for plasmid effects across 25 combinations, and for

testing plasmid and strain effects across several transconjugants, but which has limitations when applied to individual combinations). Despite this, our key findings (moderate effects on average, specificity for particular combinations) emerge from the entire dataset across the different combinations and both nutrient conditions, rather than from individual datapoints. Therefore, our key findings are likely robust.

In conclusion, our combinatorial approach revealed mostly moderate effects of clinical antibiotic resistance plasmids on biofilm formation and population growth in a range of clinical and laboratory strains. This suggests clinical AR plasmids affect bacterial phenotypes less than might be expected from previous studies with different study designs, but in a way consistent with recent studies detecting moderate effects of resistance plasmids on bacterial population growth (Alonso-del Valle et al., 2021; Vogwill and MacLean, 2015). This in turn implies that plasmids may incur only mild costs in terms of disruption of other phenotypes, which potentially contributes to their persistence in the absence of antibiotic selection.

Declaration of Competing Interest

We have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plasmid.2023.102706.

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