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Deletion of *pcnB* affects antibiotic susceptibility in resistant *Escherichia coli* by reducing copy number of ColE1-family plasmids

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Plasmids play a major role in the spread of antibiotic resistance genes in bacteria. Plasmid copy number (PCN) is often tightly regulated. In plasmids of the ColE1-type, this regulation happens by a negative feedback mechanism using an antisense RNA. Here, we employed a sequencing-based method for determining PCN to demonstrate that copy number of different ColE1-family plasmids harboring antibiotic resistance genes increases during antibiotic treatment. Further, we show that deletion of the gene *pcnB* reduces the copy number of ColE1-family plasmids in *E. coli* MG1655, which in turn results in a reduced resistance to antimicrobials of the classes aminoglycosides, β-lactams and tetracyclines. In the absence of antibiotic selection, the deletion of *pcnB* also decreased the number of ColE1-type plasmids in a bacterial population. Hence, PcnB, which polyadenylates RNA, marking it for decay, represents a potential drug and helper-drug target that could be used to reduce PCN to re-sensitize bacteria with multi-copy-number resistance-plasmids to treatment with different antimicrobials.

Antimicrobial resistance (AMR) has become a major threat to human and animal health and the increased spread of antibiotic resistance genes is primarily mediated by plasmids, which can be transferred horizontally between bacteria[1,2]. Plasmids are circular, double-stranded DNA molecules that, apart from encoding genes involved in their transmission and maintenance, often contain multiple antibiotic resistance genes[3,4]. Plasmids can replicate independently of the bacterial chromosome starting from an origin of replication (*ori*), which is one of the most important factors determining the plasmid copy number (PCN)[5,6]. The PCN, which is the average number of plasmid copies per chromosome, has a major impact on plasmid-associated traits including antibiotic resistance. Thus, the higher the copy number, the more copies of antibiotic resistance genes are inside the cell, which can result in an elevated level of resistance[3]. Hence, targeting PCN could be a possibility to re-sensitize bacteria to antibiotics.

To investigate PCN, various direct and indirect approaches have been described[7,8]. To indirectly quantify the copy number, the activity of plasmid-encoded proteins e.g. luciferases or fluorescent reporters can be assessed, assuming the premise that the PCN and the protein activity are directly proportional to each other[7–10]. Direct PCN determination methods include more traditional approaches such as dye-buoyant density gradient centrifugation combined with radioisotope labelling, and plasmid extraction followed by quantification via gel band and southern blotting analysis[10–12]. Advances in technologies have led to fluorescence in situ hybridization (FISH)-based techniques for plasmid visualization[13] and PCR-based methods including quantitative real-time PCR and Droplet Digital PCR for direct copy number investigations[7,14–16]. Recently, whole genome sequencing-based methods, which generally compare the abundance of plasmid DNA reads to chromosomal DNA reads, have been utilized for relative or absolute PCN quantification[17–22]. The advantages of sequencing-based approach are that (1) the copy number is quantified directly, (2) no toxic compounds are required during sample preparation, and (3) the methodology is less labor-intensive since no qPCR primers or reporter constructs need to be designed and validated[7,8,12].

In most plasmids, the copy number is tightly controlled, e.g. at the level of replication initiation by utilizing an RNA antisense mechanism, which has been well-studied in ColE1-type plasmids[6,23–25]. Members belonging to the ColE1-family include frequently used, medium and high-copy number cloning vectors such as pBR322, pUC19 harboring a point mutation in the pre-primer (RNA II) resulting in a higher copy number, and pACYC derivates possessing a p15A *ori* similar to ColE1 plasmids[6,26,27]. Furthermore, naturally occurring plasmids

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with ColE1-type replication systems, which include e.g. ColRNAI and Col(pHAD28) plasmids, are relatively common in nature with an estimated prevalence for all known plasmids of around 11% in *Escherichia* and up to 19% in *Klebsiella* species[28]. ColE1-type plasmids have identical or highly similar replication origins, and copy numbers are regulated by the same antisense RNA mechanism[6,26]. The mechanism involves the transcription of an unstable antisense RNA (RNA I) that is complementary to the pre-primer RNA (RNA II) needed to initiate the plasmid replication[23,25]. With an increasing copy number, the number of RNA I transcripts rises, leading to the formation of an RNA I-RNA II complex, which in turn prevents the start of the plasmid replication[23,28,29]. RNA I is produced in an approximate 100-fold excess compared to RNA II, hence a rapid turnover of this molecule, which has a short half-life of around 1.3 to 2 min, is vital to accurately detect changes in PCN per cell[25,30].

In bacteria lacking the gene *pcnB*, stability of the RNA I molecule is increased considerably with a predicted half-life of more than 15 min[25]. The deletion of *pcnB*, a gene that was first described in 1986 and named after its involvement in *p*lasmid *c*opy *n*umber[31], has frequently been reported to reduce plasmid copy number in the ColE1 family plasmids pBR322 and pACYC derivates[32–34]. The mechanism of PCN reduction in *pcnB* deletion mutants was suggested to involve an RNA regulatory component after it became evident that PcnB and the previously identified poly(A) polymerase described by Sippel in 1973 were identical[34,35]. Hence, the role of PcnB in PCN regulation has been attributed to its polyadenylating activity, where PcnB is able to polyadenylate RNA I, marking it for decay[25]. The role of the gene *pcnB* was investigated simultaneously by several groups in the 1980/90s and deletion of this gene was reported as a potentially useful cloning tool[31–34]. More recently, the interest in PcnB has shifted as it was described that more than 90% of the *E. coli* transcripts were polyadenylated to some extend by PcnB, highlighting the role of PcnB in general RNA quality control and not merely on the plasmid level[36].

The aim of the current study was to investigate how antibiotic exposure and deletion of the PCN maintenance gene *pcnB* influence the PCN in *E. coli*. Additionally, we aimed to assess the potential of *pcnB* as helper-drug target to re-sensitize bacteria with multi-copy number plasmids to antibiotic treatment. To do so, we developed a sequencing-based method to quantify PCN, followed by phenotypic analysis on *pcnB* deletion mutants harboring different ColE1-type plasmids to assess the effect of the reduced copy number on antibiotic susceptibility.

Results

Validation of the sequencing-based PCN determination method

The reproducibility of our sequencing-based method to determine PCN was tested using the well-studied medium copy number plasmid pBR322, the high copy number plasmid pUC19 and a derivate of the medium copy number plasmid, pACYC184 by comparing the estimated copy number in three technical replicates for all three plasmids. The mean PCNs of PBR322, pUC19 and a pACYC184 derivate were 15, 84 and 20 when duplicate reads were removed and 16, 116 and 21 without removing duplicate reads), with relative standard deviations of 8.2%, 7.6% and 3.5%, respectively (Supplementary Figure S1 online). The measured PCNs were similar to those previously estimated by other methods including droplet digital PCR and fluorescent microscopy for the same plasmids and their derivates[7,37–39], and the relative standard deviations suggested good reproducibility of the method.

Quantification of the PCN in WT strains and the *pcnB* mutants in the presence/absence of aminoglycoside antibiotics

To confirm and quantify previous observations that the PCN of laboratory ColE1-family plasmids is decreased in a *pcnB* deletion background[32–34], we measured the PCN of two pACYC184 derivates harboring a gentamicin (GEN) or streptomycin (STREP) resistance gene cassette (pACYC184_*aac(3)IV* and pACYC184_*strAB*)[40] using our sequencing-based quantification method. The two pACYC184 derivates were transformed independently into WT *E. coli* MG1655 and an *E. coli* MG1655 *pcnB* deletion mutant and the PCN of these resistance-plasmid containing strains was assessed in the presence and absence of ½ minimum inhibitory concentration (MIC) of the corresponding aminoglycoside. We observed an increase in the measured PCN in the presence of GEN/STREP relative to controls without antibiotics in both WT and *pcnB* mutant strains (Fig. 1). Comparing the aminoglycoside-resistant WTs to the *pcnB* deletion mutants revealed a decrease in the average PCN from 20 (21 without removing reads) in the GEN-resistant WT (Fig. 1a) and 16 (17 without removing duplicate reads) in the STREP-resistant WT (Fig. 1b) to less than 1 copy in both mutants in the absence of antibiotic pressure (Fig. 1), confirming the role of *pcnB* in plasmid copy number control[32–34]. A similar decrease in average PCN was also determined in the presence of GEN and STREP. Here, the copy number decreased from 32 copies (35 without removing duplicate reads) in WT to ~ 1 (with or without duplicate reads) in the *ΔpcnB* mutant in the GEN-resistant strains (Fig. 1a) and from 25 copies (28 without removing duplicate reads) in the WT to ~ 2 (with or without duplicate reads) in the *ΔpcnB* mutant in the STREP-resistant strains (Fig. 1b).

Phenotypic testing of WT strains and the *pcnB* mutants harboring pACYC184 derivates

To determine the effect of the reduction in PCN due to the *pcnB* deletion on antimicrobial resistance, MIC testing and growth analysis of strains were performed. MIC testing revealed that the GEN- and STREP-resistant *pcnB* deletion mutants showed a 4–8 fold decrease in MIC compared to the corresponding aminoglycoside-resistant WTs (Table 1). Furthermore, the growth analysis uncovered that under control conditions, the growth of the aminoglycoside-resistant *pcnB* mutants was slightly attenuated compared to the corresponding WTs with an average maximum growth rate μ of around 0.8 (± 0.28) OD unit * h^{-1} in the WT compared to around 0.55 (± 0.16) OD unit * h^{-1} in the *pcnB* deletion mutants (Fig. 2, a + d). Addition of 25 and 50 mg/L GEN as well as 350 and 700 mg/L STREP affected the growth of the *pcnB* deletion mutants considerably more than the WT strains (Fig. 2, b + c + e + f). The average maximum growth rate of the aminoglycoside-resistant WTs in the presence of

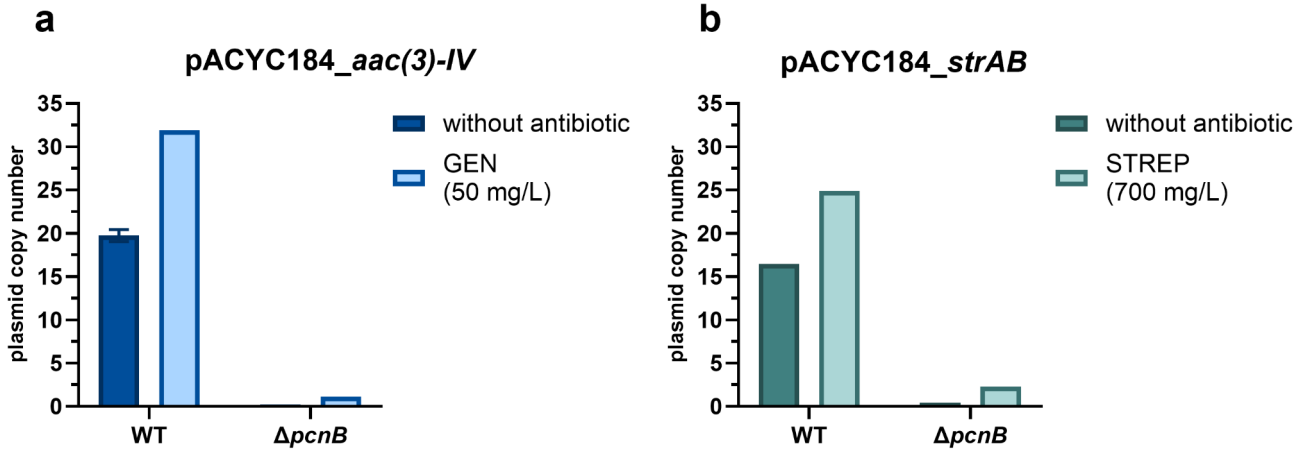

Fig. 1. Measured PCN of pACYC184 derivates in WT strains and *ΔpcnB* mutants with and without 50 mg/L GEN (**a**) and 700 mg/L STREP (**b**). The average PCN of pACYC184_*aac(3)-IV* and pACYC184_*strAB* was assessed using a sequencing-based method. The experiment was performed in one biological replicate.

E. coli MG1655 strain	Antibiotic	MIC (in µg/mL)	Fold change*
WT + pACYC184_*aac(3)-IV*	GEN	128	
ΔpcnB + pACYC184_*aac(3)-IV*	GEN	16–32	4-8
WT + pACYC184_*strAB*	STREP	2048	
ΔpcnB + pACYC184_*strAB*	STREP	256–512	4-8

Table 1. MIC of GEN and STREP in WT strains and *ΔpcnB* mutants harboring pACYC184_*aac(3)-IV* and pACYC184_*strAB*. *Fold change: MIC of WT divided by MIC of corresponding *ΔpcnB*.

50 mg/L GEN and 700 mg/L STREP and were around 0.74 (± 0.25) and 0.46 (± 0.12) OD unit * h^{-1}, while in the corresponding *pcnB* deletion mutants the maximum growth rate was decreased to 0.12 (± 0.11) and 0.17 (± 0.08) OD unit * h^{-1} respectively, reflecting a 64 to 84% decrease (compared to an around 35% decrease without antibiotic). While the lag phase was around 3 h in the WT strains (with and without antibiotics) as well as in the *pcnB* deletion mutants (without antibiotics), the lag phase increased to more than 10 h in the *pcnB* deletion mutants in the presence of 25/50 mg/L GEN and 350/700 mg/L STREP. Hence, increasing concentrations of aminoglycosides led to a prolonged lag phase followed by a slower and more variable growth of the *pcnB* deletion mutants (Supplementary Figure S2 online) compared to the WT strains (Fig. 2, b + c + e + f).

Phenotypic testing of WT strains and the *pcnB* mutants harboring other ColE1-type plasmids

To investigate whether the deletion of *pcnB* also resulted in a reduced MIC compared to the WT when resistance was encoded from other ColE1-type plasmids, the well-studied plasmids pUC19 and pBR322 as well as two naturally occurring ColE1-type plasmids were transformed independently into the MG1655 WT and the *pcnB* deletion mutant. pUC19 encodes ampicillin (AMP) resistance through β-lactamase gene *bla*[41], and pBR322 possesses the same AMP resistance gene as well as the tetracycline (TET) resistance gene, *tet(A)*, encoding for a TET efflux pump[42,43]. MIC testing revealed an 8-fold decrease in MIC to AMP in the *pcnB* deletion mutants harboring pUC19 compared to the corresponding WT, and Δ*pcnB*_pBR322 displayed a 16-fold and an 8-fold reduction in MIC to AMP and TET, respectively, compared to the corresponding WTs (Table 2).

The natural ColE1-type plasmid ColRNAI_*aph(3')-Ia* was originally isolated from a clinical *E. coli* strain found in pigs and encodes for an aminoglycoside O-phosphotransferase conferring resistance to neomycin (NEO) and kanamycin (KAN)[44]. Assessing the MIC of these aminoglycosides uncovered 2-4 fold reduction to NEO and a 2-fold reduction to KAN in the *pcnB* mutant compared to the WT (Table 2). The naturally occurring plasmid Col(pHAD28)-*tet(A),* which was also isolated from an *E. coli* strain found in pigs, encodes for a TET efflux pump[45]. This Col(pHAD28)-*tet(A)* plasmid was sequenced and assembled (NCBI submission ID 2875940). Testing the MIC of TET revealed a 4-fold reduction in the *pcnB* mutant compared to the WT (Table 2). Furthermore, the absolute copy number of this Col(pHAD28)-*tet(A)* plasmid in the WT and the *pcnB* deletion mutant background was quantified. In the absence of TET, on average 7.4 plasmid copies were present in the WT, while on average only every second *pcnB* deletion mutants harbored a plasmid (average copy number: 0.5). In the presence of 128 mg/L TET, the PCN in both strains increased, however unexpectedly more in the *pcnB* mutant than in the corresponding WT strain (Supplementary Figure S3 online).

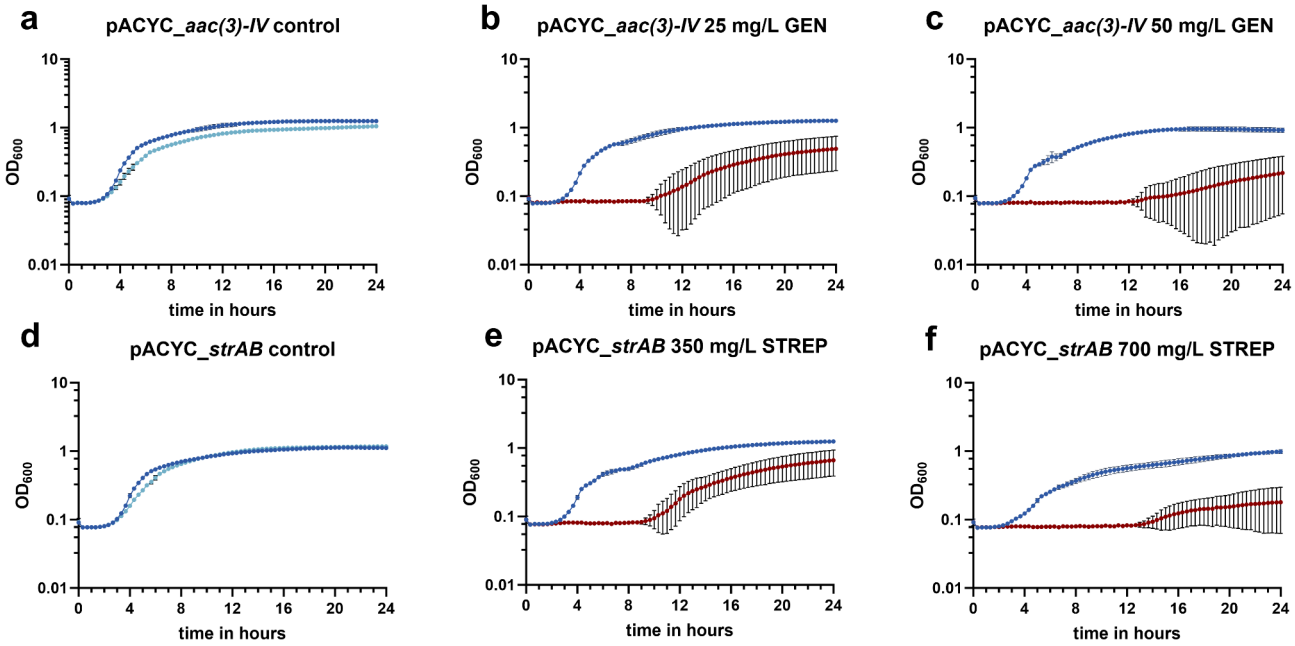

Fig. 2. Growth curves of the WT strains and *ΔpcnB* mutants harboring pACYC184_*aac(3)-IV* (**a**–**c**) and pACYC184_*strAB* (**d**–**f**) in the absence (**a**, **d**) and presence of 25/50 mg/L GEN (**b**, **c**) and 350/700 mg/L STREP (**e**, **f**). The growth of the GEN-resistant WT (dark blue) compared to the growth of the GEN-resistant *ΔpcnB* (light blue/red) in LB is shown under control conditions (**a**), in the presence of 25 mg/L GEN (**b**) and in the presence of 50 mg/L GEN (**c**). The growth of the STREP-resistant WT (dark blue) compared to the growth of the STREP-resistant *ΔpcnB* (light blue/red) in LB is shown under control conditions (**d**), in the presence of 350 mg/L STREP (**e**) and in the presence of 700 mg/L STREP (**f**). The growth variation between the replicates for the *ΔpcnB* mutants is shown in Supplementary Figure S2 online. The experiment was performed in four biological replicates with three technical replicates each.

E. coli MG1655 strain	Antibiotic	MIC (in µg/mL)	Fold change*
WT + pUC19	AMP	16,384	
ΔpcnB + pUC19	AMP	2048	8
WT + pBR322	AMP	16,384	
ΔpcnB + pBR322	AMP	1024	16
WT + pBR322	TET	64–128	
ΔpcnB + pBR322	TET	8–16	8
WT + ColRNAI_*aph(3')-Ia*	NEO	1024	
ΔpcnB + ColRNAI_*aph(3')-Ia*	NEO	256–512	2-4
WT + ColRNAI_*aph(3')-Ia*	KAN	4096	
ΔpcnB + ColRNAI_*aph(3')-Ia*	KAN	2048	2
WT + Col(pHAD28)_*tet(A)*	TET	256	
ΔpcnB + Col(pHAD28)_*tet(A)*	TET	64	4

Table 2. MIC testing results in MG1655 WT and MG1655 *ΔpcnB* harboring pUC19, pBR322 or ColRNAI_*aph(3')-Ia*. *Fold change: MIC of WT divided by MIC of corresponding *ΔpcnB*.

Homology of PcnB to human and other bacterial proteins

The gene *pcnB* encodes for the protein PcnB which consists of 465 amino acids (NP_414685.4). We compared the amino acid sequence of PcnB to the human proteome in silico and confirmed that PcnB did not share significant homology with human proteins. Comparison to other bacterial proteins revealed that PcnB (with 100% identity) was present in other members of the *Enterobacteriaceae* family including multiple other *E. coli* strains, as well as in strains from *Shigella* and *Klebsiella* species. Furthermore, PcnB variants with more than 80% sequence identity were identified in other bacterial species belonging to genera such as *Salmonella*, *Cronobacter, Lelliottia, Enterobacter, Kosakonia, Leclercia, Siccibacter, Pluralibacter, Klebsiella, Citrobacter, Cedecea, Mangrovibacter, Jejubacter, Pantoea, Erwinia* and *Nissabacter* (Supplementary Table S1 online).

Discussion

In the current study, we have investigated the PCN of different ColE1-type plasmids in an *E. coli* MG1655 *pcnB* deletion mutant versus the WT in the presence and absence of antibiotics utilizing a sequencing-based quantification method. Furthermore, we have conducted phenotypic analysis including growth curves and MIC testing to examine the consequences of the reduced copy number of ColE1-type plasmids observed in the *E. coli pcnB* deletion mutants on antibiotic susceptibility.

To quantify PCN, a sequencing-based approach, which allowed absolute quantification of PCN was implemented. In this method we assumed that one chromosome was present per bacterial cell and the number of plasmid reads was set in relation to the number of chromosomal reads similar to previously published sequencing-based methods[17–19]. Our PCN measurements for the well-studied ColE1-type plasmids pUC19, pBR322 and a pACYC184 derivate were in a similar range as previously published PCNs determined by different methods[7,37,38,46], confirming that our sequencing-based method was suitable for relative and absolute copy number quantification. Since the technical triplicates of these three plasmid quantifications showed a similar copy number with very low relative standard deviation, we performed quantification of PCN in the following experiments based on one replicate. As part of the analysis, potential duplicate reads were filtered out, which could lead to an underestimation of the PCN in high copy number plasmids. Of note, this underestimation appears to be more pronounced when the PCN is larger, with around 28% for PCN around 100 (pUC19) compared to less than 6% for a PCN around 20 (pBR322 and pAYCY184 derivates, Supplementary Table S4 online).

After verification of the sequencing-based approach, we used this method to compare the PCN of two pACYC184 derivates (encoding aminoglycoside resistance genes) and a naturally occurring ColE1-family plasmid (Col(pHAD28)_*tet(A)*) in *E. coli* MG1655 WT strains and *pcnB* deletion mutants with and without ½ MIC of the corresponding antibiotic. Overall, we demonstrated that the PCN is reduced in the *pcnB* deletion mutants compared to the WT strains in the absence of antibiotics. This confirmed previous works reporting that deletion of *pcnB* affects copy number of ColE1-type plasmids[31–33]. Furthermore, we observed that the PCN was generally increased during antibiotic exposure which has also been suggested previously e.g. for pBR322 under chloramphenicol (CHL) exposure[47]. One possible explanation for this observation could be that antibiotics create a strong selective pressure, leading to the selection of clones with higher copy number since it has been shown that PCNs in a bacterial population fluctuate[16,37,48] and that sub-lethal antibiotic concentrations are able to eliminate cells harboring fewer resistance plasmids[48]. Furthermore, previous studies have demonstrated that antibiotic exposure leads to altered gene expression (including genes associated with bacterial stress response) as well as increases conjugation frequency[49,50], raising the question whether an active mechanism of antibiotics might also be involved. Hence, to determine if antibiotic exposure directly (over an active mechanism) or indirectly (over selection pressure) increases the PCN in a bacterial population requires further investigation.

In the absence of antibiotics, the average PCN in the *pcnB* deletion mutant decreased to less than 0.5 plasmids per cell indicating that most *pcnB* mutants appeared to lose the plasmids without antibiotic pressure which fitted to a previous assumption[32], although the PCN was not quantified. In the presence of ½ MIC of the corresponding antibiotic, the pACYC184 PCN increased to an average of 1–2 plasmids per cell in the *pcnB* mutant which may just reflect, that cells with plasmids, which carry the resistance genes, are selected. The reason for the PCN reduction in the *pcnB* deletion mutants has been attributed to the accumulation of RNA I, which has an increased stability when it is not polyadenylated by PcnB, and ColE1-type plasmids employ this RNA I as a negative control mechanism to regulate their PCN[25,51]. Even though some observations concerning the PCN reduction were made decades ago and the expression of the β-lactamase encoded by pBR322 has been used to estimate copy number, deletion of *pcnB* has merely been reported as a useful tool for cloning[31,33].

One focus of the current study was to assess the potential of PcnB as helper-drug target to re-sensitize *E. coli* harboring ColE1-family plasmids to antibiotics. In phenotypic analysis, we demonstrated that the growth of an aminoglycoside-resistant *pcnB* deletion was more affected than WT at ¼ and ½ MIC concentration of the aminoglycoside, since the lag phase was extended (from around 3 to more than 10 h) and the maximum growth rate was decreased more compared to the corresponding WT. Furthermore, a more variable/fluctuating growth in the *pcnB* deletion mutants (compared to the WT) was observed as evident by the standard deviation. Plotting each of the 12 replicates of the *pcnB* deletion mutant growth individually revealed that this variation comes from both biological and technical replicates (Supplementary Figure S2 online). A potential explanation for this more variable growth could be that each cell in the starting *pcnB* deletion population has a different number of plasmids (including varying rates of plasmid loss) requiring further experiments for clarification. However, at the start of the experiment, each cell harbored at least one plasmid since all strains were grown on selective media to avoid a plasmid loss before the experiment. Furthermore, we showed that a reduction in PCN of different ColE1-family plasmids (caused by the deletion of *pcnB*) resulted in a 2–16-fold increase in antibiotic susceptibility to aminoglycosides, AMP and TET. A limitation of MIC testing is that it is a semiquantitative measure which can vary between replicates (2-fold being the acceptable range). To overcome this problem, MIC testing of the WT and corresponding *pcnB* deletion mutant were always performed together in this study allowing a direct comparison expressed as fold change of mutant compared to WT rather than change in actual MIC. For a more quantitative assessment of resistance, further experiments including half maximal inhibitory concentration and growth rate measurements could be performed.

Notably, the relationship between PCN/resistance gene dosage and MIC is not directly proportional or linear, since a 20–30-fold reduction in PCN resulted in a 4–8 fold decrease in MIC, which is in line with the previous observation that an increased resistance gene dosage due to increased PCN does not always correlate with its measured MIC[52]. A potential reason for this could be that additional levels of regulation on the RNA or protein level are involved, requiring further investigation. The attenuated growth as well as the reduction in MIC in the *pcnB* deletion mutant background highlight the potential of PcnB as helper-compound target candidate. Except for one study where *pcnB* was reported to play a role in MarA-mediated resistance in a sensitive *E. coli* BW25113

(harboring no additional resistance genes)[53], *pcnB,* to the best of our knowledge, has not previously been linked to AMR in antibiotic-resistant bacteria.

In addition to the function as helper-drug target to increase susceptibility to antibiotics, our results indicated that targeting *pcnB* could potentially be used to eliminate/reduce the number of ColE1-type plasmids in the absence of antibiotics. We have preliminary investigated this by studying the copy number of the plasmid Col(pHAD28)_*tet(A)*, which decreased by 69% within 48 h in the *pcnB* deletion mutant while the PCN in the WT strain decreased by only 11% in the absence of antibiotic pressure (data not shown). However, additional confirmation of this observation by performing biological replicates are needed, and since the observation indicates that PcnB could be used as a potential drug target to reduce the frequency of ColE1-family plasmids in a bacterial population in the absence of antibiotic pressure, further experiments using different ColE1-family plasmids are also indicated. Furthermore, our results suggested that PcnB is a promising (helper)-drug target as this gene (1) is not essential in *E. coli*[34,40], (2) has no homology to human proteins and (3) appears to be conserved among *E. coli* strains and other *Enterobacteriaceae*. Similar protein sequences were also identified in other bacteria, however, with less homology.

A limitation of the current study is that all experiments were performed in a *pcnB* deletion mutant in the *E. coli* laboratory strain MG1655 and in LB media. To validate PcnB as helper-drug target, different bacterial strains with *pcnB* deletions and additional clinically relevant plasmids with antisense RNA control mechanisms should be analyzed and in vivo models that are closer to infection settings should be used. Furthermore, the relationship between *pcnB*, PCN and level of antibiotic resistance seems to be more complex as evident by the differences in the MIC fold changes (for plasmids with several resistance genes) and differences in copy number during antibiotic exposure. Previous research has shown that the interplay between plasmid dynamics and gene expression variability is complicated and contributes to resistance, explaining why higher PCNs do not necessarily lead to increased MIC values[48]. Additional factors such as (1) resistance genes, (2) choice of antibiotic, (3) antibiotic exposure and (4) additional levels of regulation seem to influence the copy number as well as the antibiotic susceptibility and further research is needed to clarify the underlying relationships.

In conclusion, we show that PcnB is a potential helper-drug target, since deletion of this gene reduces the copy number of ColE1-family plasmids which in turn results in an increased susceptibility to different classes of antibiotics. Additionally, PcnB influences the stability of ColE1-family plasmids over time and could therefore be employed as a drug target candidate to reduce ColE1-type plasmid frequency in a bacterial population. Furthermore, we validated our sequencing-based method as a useful tool to quantify and investigate changes in PCN. Our study provides an insight into the factors that influence PCN and how these can be utilized to re-sensitize bacteria with multi-copy number plasmids to antibiotics.

Methods

Bacterial strains and cultivation

E. coli K12 MG1655[54] was the main strain used in the current study. All strains and plasmids employed in the study are listed in Supplementary Table S2 online. All MG1655 wild type (WT) strains and all *pcnB* deletion mutants harboring different ColE1-type plasmids are referred to as WT strains and *pcnB* deletion mutants. Strains were cultivated in Luria Broth (LB) Lennox with shaking or on LB agar plates (both Becton Dickinson, Albertslund, Denmark) overnight at 37 °C; apart from strains harboring the temperature sensitive plasmids pKD46 or pCP20, which were cultivated at 29 °C. When necessary, antibiotics were added to the media including 100 mg/L AMP, 50 mg/L STREP, 20 mg/L GEN, 50 mg/L NEO, 20 mg/L TET or 50 mg/L CHL (Sigma-Aldrich, Copenhagen, Denmark).

Plasmid isolation, sequencing and assembly

All plasmids used in this study (Supplementary Table S2 b online) were isolated using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Roskilde, Denmark) from overnight cultures of the original strains (Supplementary Table S2 a online) according to manufacturer's instructions. After isolation, the plasmid Col(pHAD28)-*tet(A)*, was sequenced and assembled using Plasmidsaurus (Cologne, Germany). The plasmid sequence is available under GenBank PQ495701.

Preparation of electrocompetent cells and transformation

E. coli MG1655 electrocompetent cells were prepared in essence as described previously[40,55]. Briefly, *E. coli* MG1655 was grown in LB medium overnight, followed by a 1:100 dilution in LB media and incubated until an optical density (OD_{600}) of 0.5–0.8 was reached. The cells were centrifuged and washed four times: with 1 × and 0.5 × volume of cold deionized water, twice with 0.05 × volume of 10% ice-cold glycerol and finally resuspended in 0.002 × volume of 10% ice-cold glycerol. Aliquots of electrocompetent cells were either used directly or stored at − 80 °C until further use.

For electroporation, 1 µL isolated plasmid and 50 µL of electrocompetent cells were mixed in a 2 mm cuvette and an Eporator® electroporator (Eppendorf, Hamburg, Germany) set to 2.5 kV was used, essentially as described previously[55]. Afterwards, the transformed cells were recovered in 900 µL pre-warmed Super Optimal broth with Catabolites repression (SOC) medium (Thermo Fisher Scientific, Roskilde, Denmark) and incubated with shaking at 37 °C (or 29 °C for strains with temperature sensitive plasmids) for 1 h. Then, 1 and 10 µL of the cells were plated on LB agar plates with the appropriate antibiotic and incubated at 37 °C (or 29 °C) overnight. The next day, colony PCR with specific primers (Supplementary Table S3 online) of eight to ten antibiotic-resistant colonies was performed to verify the presence of the correct plasmid.

Construction of plasmid-containing MG1655 WT strains and *pcnB* deletion mutants

The Lambda Red recombination system was used to construct a Knock-out (KO) mutant of *pcnB* in *E. coli* MG1655 utilizing the plasmids pKD46, pKD3 and pCP20, essentially as described previously[56,57]. All primers used for the KO constructions are shown in supplementary Table S3 online. To verify the presence of the CHL resistance cassette which replaced *pcnB*, a colony PCR was performed (primers are listed in supplementary Table S3 online). The helper-plasmid pCP20 encoding a FLP recombinase was employed to construct a clean *pcnB* deletion mutant as stated previously[56,57] and the correct deletion was confirmed by colony PCR with the same primers. Afterwards, the different plasmids (pACYC derivates-STREP/GEN, CHL resistances; pUC19-AMP resistance; pBR322 – TET and AMP resistances; ColRNAI_*aph(3')-Ia* – NEO/KAN resistance; Col(pHAD28)_*tet(A)* – TET resistance) were transformed independently into *ΔpcnB* and the WT as described above and all resulting strains are listed in supplementary Table S2 online.

PCN determination

Bacterial samples were prepared essentially as described previously[40]. Briefly, 0.5 McFarland standards (approximately 10^8 colony forming units /mL) of the plasmid-containing strains were prepared. One hundred µL of each McFarland standard were added to 10 mL of LB media, either without antibiotic (control) or containing the corresponding antibiotic (treatment). Each mixture was incubated at 37 °C (shaking) for 24 h, with the treatment flask containing a final concentration of 700 mg/L STREP, 50 mg/L GEN or 128 mg/L TET.

Subsequently, 400 µL of each bacterial sample from the control and treatment flasks were harvested, treated with RNase, and DNA was extracted utilizing an automated Maxwell® RSC Cultured Cells DNA purification kit and instrument, according to manufacturer's instructions (Promega, Madison, USA). To test the reproducibility of the method, 400 µL of the control samples harboring pBR322, pUC19 and the derivate plasmid, pACYC184_*aac(3)-IV,* were prepared in triplicates. Quantification of the extracted DNA was performed using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Roskilde, Denmark), while DNA quality was measured with NanoDrop. DNA samples of appropriate quality (showing an OD 260/280 ratio of ≥ 1.8–2.0 and an OD 260/230 ratio of ≥ 1.8–2.2) were chosen. The isolated DNA was used to prepare a standard Illumina 150 bp paired-end library following manufacturer's protocol. DNA sequencing was performed by Illumina NovaSeq 6,000 sequencing technology at Eurofins Genomics (Constance, Germany). All generated raw sequencing reads are available in the NCBI Sequence Read Archive under BioProject PRJNA1165952.

As illustrated in supplementary Figure S4 online, the relative mapping-depth (number of reads per unit) between plasmid DNA and chromosomal DNA of a given sequence sample was used to estimate the PCN in that strain. To obtain mapping-depth-values, sequencing data were aligned to the respective reference sequences of the chromosome or plasmid (detailed in the supplementary Table S4 online). Pre-processing of raw reads and mapping for reads were done using an in-house Snakemake[58] pipeline (https://github.com/china-fix/rq-count_v1). In brief, the raw reads underwent initial preprocessing using fastp version 0.12.4[59] to obtain clean reads. These were then aligned to the corresponding chromosome and plasmid reference sequences using the Burrows-Wheeler Aligner (BWA) version 0.7.17[60]. Subsequently, the outputs were processed in two ways: either directly proceeding to the next step without filtering or by first filtering out potential duplicate reads using the Picard Toolkit (version 2.18.7, Broad Institute, Boston, USA). For both approaches, the mapping depth at each base pair was determined using samtools (version 1.17)[61] and the results were output as tab-separated files. To obtain the mean and median mapping-depth of sequence of the chromosome or plasmid, the tab files were processed and calculated by an in-house Python script (https://github.com/china-fix/rq-count_v1/blob/main/scripts/Get_mapping_summary.py). In short, the script first reads all the mapping-depth values, i.e. mapping-depth for every base pair. These mapping-depth-values for each base pair were expected to follow a normal distribution, and the mean and a 90% confidence interval was calculated for each compartment (plasmid or chromosome). Ten percent outlier values were excluded to form a new dataset, and this was then used to calculate the mean and median mapping-depth-values for the plasmid and the chromosome.

Antimicrobial susceptibility testing

The MIC of STREP, GEN, NEO, KAN, AMP and TET against the strains under study was determined by broth micro-dilution method following the CLSI guidelines[62]. MIC testing was conducted in at least three biological replicates, each consisting of two technical replicates. The MIC of the WT and the corresponding *pcnB* deletion mutant were always assessed in parallel to allow a direct comparison.

Bacterial growth curves

Bacterial growth experiments were conducted in four biological replicates (with three technical replicates each) using a Bioscreen C automated system (Thermo Labsystems) basically as described previously[40]. Shortly, a 0.5 McFarland suspension of each strain was prepared and 100 µL of the bacterial suspension was added to 10 mL LB media. Afterwards, 100 µL of LB containing bacteria were mixed with 100 µL of LB media with or without the corresponding antibiotic (final concentration: 350 and 700 mg/L STREP, 25 and 50 mg/L GEN, which corresponded to ¼ and ½ MIC of the aminoglycoside-resistant WT strains) in a 100-well honeycomb plate. The OD_{600} of the bacterial culture was measured every 20 min under continuous, medium shaking for 24 h at 37 °C. Of note, due to the absence of sample dilution in the Bioscreen C automated system, OD_{600} measurements exceeding 1.0 may not accurately represent cell density.

In silico homology analysis of PcnB with other proteins

To assess if PcnB has a homologue in the human proteome, the amino acid sequence of PcnB was extracted from NCBI (NP_414685.4) and compared to the human proteome with NCBI Protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi using *Homo sapiens* taxid: 9606 with an E-value cutoff of 10×10^{-10}[63]) in essence as described

previously[64]. To identify proteins with more than 80% sequence identity in other bacterial species, the NCBI-pBlast tool with the nr_clustered (experimental) database was used (Supplementary Table S1 online).

Statistical analysis and data presentation

The average PCN, the standard deviation of the mean copy number and the standard deviation divided by the mean copy number and expressed as percentage (relative standard deviations) were calculated using Excel. The average OD_{600} shown in the growth curves was calculated in GraphPad Prism 10 by using the average OD_{600} of four biological replicates (consisting of three technical replicates) with the corresponding ± standard deviation. The maximum growth rate (including the corresponding standard deviation) was calculated in Excel using the average OD_{600} (at a specific time point) and following formula: maximum growth rate = $(\ln(OD_{600}\_2) - \ln(OD_{600}\_1)) / (time\_2 - time\_1)$[65,66]. The lag phase was defined as the period during which the maximum growth rate remained below 0.2 OD unit * h^{-1}. Growth curves as well as PCN measurements were illustrated with GraphPad Prism 10 (GraphPad Software, San Diego, USA).

Data availability

The data shown in this study is available in the article and supplementary materials. Raw sequencing data is available under accession no. PRJNA1165952.

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Author contributions

S.M.W., X.F., A.H.-F. and J.E.O.: Conceptualisation and methodology; S.M.W., X.F.: software and investigation: ; S.M.W., X.F. and J.E.O.: analysis; S.M.W: visualisation:; S.M.W: writing—original draft preparation; X.F., A.H.-F. and J.E.O.: writing—review and editing; A.H.-F. and J.E.O.: supervision; J.E.O.: project administration and funding acquisition; All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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