# Integron-associated structures underlie the accumulation of antibiotic resistance genes on plasmids beyond cassette arrays

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**Abstract**

The emergence and spread of multidrug-resistant bacteria posing serious threat to global public health has been a focus for many studies. Coexistence of integrons and antibiotic resistance genes (ARGs) has been widely observed in various environments. It has long been considered that most correlated ARGs were carried by integron gene cassettes. In this study, we show, for the first time, evidence that integron presence is linked to the accumulation of multiple ARGs beyond their cassette arrays, resulting in the formation of multi-ARG plasmids. By analyzing 1102 plasmid sequences retrieved from PLSDB RefSeq across incompatibility groups, host genera, and plasmid sizes, we show that the increased ARGs on integron-positive plasmids included not only ARGs present in integron cassette arrays but also those located outside integron regions. These effects were consistently observed across diverse incompatibility groups, host genera, and plasmid size ranges. Aminoglycoside resistance genes were found to be the most abundant both inside and outside integron regions, while beta-lactam, tetracycline, macrolide, and quinolone resistance genes were mostly found outside integron regions on integron-positive plasmids. We also observed the same ARG accumulation on integron-positive plasmids and lack of ARG accumulation on integron-negative plasmids from the same sampling sites by analyzing 32 long-read sequenced plasmids of *Escherichia coli* isolates collected from human and pig wastewater. This finding can reject the alternative explanation that integrons were co-selected with multi-ARG plasmids under the same antibiotic pressure. Our findings provide insights into the structural contexts underlying the accumulation of multiple ARGs on plasmids, which may inform new directions for future antimicrobial resistance surveillance and mitigation strategies.

**Keywords**

Integron, antimicrobial resistance, plasmid, mobile genetic element

# 1 Introduction

The global spread of antimicrobial resistance (AMR) poses an increasing threat to public health (Naghavi et al., 2024). The emergence and spread of multi-drug resistant "superbugs" increase the morbidity and mortality rate of bacterial infections, as well as increasing healthcare costs and antibiotic usage (Chandrasekhar et al., 2024; van Duin & Paterson, 2016). Many studies have found co-existence of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) (Johansson et al., 2023; Wan et al., 2025; Zhu et al., 2025). MGEs can efficiently spread across different bacterial strains, species, or even genera, making antibiotic resistance genes (ARGs) carried by such elements spread. By understanding where the ARGs are located and with how MGEs facilitate ARG transmission, intervention strategies can be developed and applied to slow down the spread of AMR.

Integron is one of the MGEs whose presence was strongly linked to multidrug-resistant bacteria (Nadella et al., 2022; P. Wang et al., 2020; Zhang et al., 2025), because integrons are well-known for capturing ARGs into their cassette arrays. Integrons can be detected in both anthropogenic and natural environments. Many environmental surveillance studies found strong correlation between integron and aminoglycoside, beta-lactam, chloramphenicol, and trimethoprim resistance genes, making integrons important indicators for environmental ARG surveillance (R. An et al., 2023; Ma et al., 2017; Martini et al., 2018; Tavares et al., 2024). The presence of integron was also found in many multidrug-resistant bacteria in both clinical and non-clinical settings (Adeniji et al., 2022; Jiang et al., 2025; Vásquez-Ponce et al., 2022; X. Wang et al., 2022). In global-scale network analyses, integrons were often determined as important nodes for ARG spread and gene exchange (X. Wang et al., 2024; Y. Wang & Dagan, 2024). Under the stress of antibiotics, integron could reshuffle cassettes to accelerate the evolution of *Pseudomonas aeruginosa* to gain resistance (Souque et al., 2023). A previous large-scale study found association between integrons and ARG carriage on plasmids, however, this study did not quantify the total number of ARGs per plasmid or distinguish whether these ARGs occurred inside or outside integron cassette arrays (Orlek et al., 2023). In addition, there are mismatches in ARG-integron co-existence and the ARGs that were often found on integron cassette arrays. For example, co-existence of tetracycline resistance genes and integrons has been widely found in various environmental samples (Agersø & Sandvang, 2005; Belaynehe et al., 2018; Huyan et al., 2020; Ling et al., 2013; Ma et al., 2017), but these genes were rarely identified in integron cassette arrays (Ali et al., 2024; X. L. An et al., 2022; Zhang et al., 2025). Although certain tetracycline resistance genes and integrons can co-occur within the same transposon, the corresponding transposon structures were highly diverse and could not explain all instances (Huyan et al., 2020; Liu et al., 2025; Shi et al., 2021).

In this study, we investigated whether the presence of integrons correlates with broader plasmid-level ARG accumulation. We analyzed 1102 ARG-carrying plasmids from PLSDB RefSeq and 32 plasmids isolated from environmental sources. Our results reveal a previously unquantified pattern that integron-carrying plasmids consistently harbor significantly more ARGs in regions outside the integron itself across species and incompatibility groups, suggesting that integrons may act as anchoring structures for plasmid-wide multidrug resistance expansion.

## 2 Methods

### 2.1 Plasmid sequence retrieval and annotation

The sequences of the plasmids analyzed in this study were retrieved from PLSDB (data version: 2024_05_31_v2) (Molano et al., 2025; Schmartz et al., 2022). Three-thousand two-hundred and eleven circular RefSeq plasmid sequences with annotated incompatibility groups were downloaded based on their accessions. The genes on these plasmids were annotated by BV-BRC Genome Annotation using annotation recipe “Bacteria / Archaea” and taxonomy name “Bacteria (2)” (Brettin et al., 2015; Shukla et al., 2013). After annotation, 1102 plasmids had at least one ARG annotated (data available upon reasonable request). The integron regions, including complete integron, CALIN, and In0, were identified by IntegronFinder 2.0 for these 1102 ARG-carrying plasmids (Néron et al., 2022). The ARGs located inside and outside of integron regions were determined by customized Python code (code available upon reasonable request). ARGs with at least one base-pair overlapping with the integron region were identified as inside, while no overlapping was identified as outside. The presence of IS26 insertion sequences on the plasmids was determined by BLAST using the GenBank accession X00011.1 as the query sequence (Wheeler & Bhagwat, 2007). Plasmids with hits ≥80% identities and at least 500 bp matched lengths were considered to contain the IS26-like insertion sequences.

### 2.2 Statistical analysis on number of ARGs

To compare the numbers of ARGs on plasmids in different categories, normality test was first performed to determine whether the number of ARGs on plasmids were normally distributed. Two-sample Komogorov-Smirnov test was used to assess whether the distributions of the ARG numbers on integron-positive plasmids were significantly greater than those on integron-negative plasmids in pairwise. When more than two categories were compared with each other, Kruskal-Wallis ANOVA was used to assess whether the number of ARGs were distributed significantly differently among the categories. The statistical analyses were conducted on Origin 2024.

### 2.3 Plasmid similarity analysis

The similarity of the 3211 PLSDB plasmids was assessed by calculating pairwise average nucleotide identities (ANI) using FastANI with minimum fraction of 0.5 (Jain et al., 2018). Cytoscape was used to plot networks based on the FastANI outputs (Shannon et al., 2003). A cutoff of ANI >99.5% was used to find plasmids that share highly similar sequences. Sixty-five plasmids that were clustered in the network with various numbers of ARGs and both integron-positive and integron-negative members were selected for a more detailed analysis on the ARGs on integron-positive and integron-negative plasmids.

### 2.4 Environmental sample collection and processing

Weekly-composite wastewater samples from the five human communities in two towns (Town A and Town B) were taken from manholes using automatic water samplers (Teledyne ISCO). The pork plant wastewater samples were collected at the pork plant wastewater outlet. The pork plant is in Town B. The samples were stored in sterile sampling bags during sampling and transportation (Fisherbrand). Once the sampling is completed, 20 mL of 2.5 M $MgCl_2$ were added to the samples on-site for flocculation. Then, the sampling bags were sealed and transported to the laboratory in coolers filled with ice-packs within

three hours. The samples were stored at 4 ℃ before the same-day pre-processing.

The samples were pre-processed in biosafety cabinets by filtering 10-50 mL well-mixed wastewater through 0.45 μm mixed cellulose ester (MCE) membrane filters (Millipore). The blank control was made by filtering 50 mL of molecular biology grade water. After filtering, the filters were cut into quarters. To be able to maintain bacteria viability, each quarter was merged in LB broth with 25% glycerol in a cryo-vial, pre-cooling at -20 ℃ for one hour, and stored at -80 ℃ before *Escherichia coli* isolation.

### 2.5 *E. coli* isolation and characterization

*E. coli* was isolated by Levine Eosin Methylene Blue (EMB) agar plates. Before isolation, the filters were defrosted at 37 ℃ for 30 minutes. Then, the filters were placed onto EMB plates with the sediment side attached to the agar. The plates were incubated at 37 ℃ for maximum 24 hours. After incubation, the colonies were re-streaked onto new EMB plates for another batch of incubation until round-shaped single colonies with dark purple color and green metallic sheen were present. Then, the single colonies with desired morphologies were purified by re-streaking onto EMB agar plates and incubating for three times to eliminate chance of getting mixed colonies. Blank control was included in every plate incubation steps with an EMB agar plate streaked by phosphate buffered saline (PBS). A single colony from each purification plate was enriched in cryo-vials with LB broth overnight at 37 ℃ with a shaking speed of 250 RPM to make frozen stocks. The blank control of the enrichment step was a cryo-vial with LB broth dipped by a clean inoculating loop. After enrichment, the bacteria culture was mixed with an equal volume of 50% glycerol and stored at -80 ℃ before downstream analyses.

16S rRNA gene Sanger sequencing was performed to determine whether the isolated strains were *E. coli*. Bacterial genome DNA were extracted by DNeasy Blood and Tissue Kit (QIAGEN). The bacteria cells for DNA extraction were obtained by re-streaking the frozen stock onto EMB plates once and enriching in LB broth as described above. All extraction steps followed the instruction provided by the manufacturer. All final DNA extracts were eluted with 200 μL AE buffer in the extraction kit. One extraction blank was inoculated with nothing and extracted using the abovementioned method. The DNA extracts were stored at −20 °C before the PCR of the 16S rRNA gene.

The reaction volume of PCR was 25 μL, containing 12.5 μL Taq 1x Master Mix (New England Biolabs), 1.25 μL forward primer(5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'), 1.25 μL reverse primer(5'-GTCTCGTGGGCTCGGAGATGTGTATAAAGACAGCCTACGGGNGGCWGCAG-3'), 2 μL DNA sample and 8 μL nuclease-free water. The thermal cycle included a pre-denaturation stage of 95 ℃ for 15 min, a PCR stage with 40 cycles of 95 °C for 20s, 55 °C for 45s, and 68 °C for 45s, and a final extension stage at 68 °C for 5 min. After PCR, Exo-CIP™ Rapid PCR Cleanup Kit (New England Biolabs) was used to clean up the primers and polymerases. The PCR product was quantified by Qubit dsDNA BR Assay Kit (Thermo Fisher). Then, the DNA samples were submitted to Roy J. Carver Biotechnology Center for Sanger sequencing. BLAST was conducted to compare the Sanger sequencing results to the nucleotide database on NCBI, and the bacteria strain was determined as *E. coli* if the top match of the BLAST result was *E. coli*.

We used the microdilution method following Clinical and Laboratory Standards Institute (CLSI) M100-Ed33 for determining the minimum inhibitory concentration (MIC) values of each isolate to ampicillin, chloramphenicol, ciprofloxacin, tetracycline, gentamicin, trimethoprim, and sulfamethoxazole (data available upon reasonable request). Before the microdilution experiment, *E. coli* were re-streaked from the frozen stocks onto LB agar plates and incubated at 37 ℃ for maximum 24 hours. The inoculum was prepared by dissolving colonies to molecular biology grade water and adjusting the OD600 value to 2.0 ± 0.2. The microdilution experiment was conducted on 96-well plates in Mueller Hinton Broth 2 (MHB2) with serial-diluted antibiotics. All antibiotics were 2-fold serial diluted for ten dilution levels above and below the MIC threshold of each antibiotic listed by CLSI M100-Ed33. The MIC values were tested in duplicates for each *E. coli* strain. The final concentration of bacteria cells in each well was adjusted to $5\times10^5$ CFU/mL. A sterile (negative) control was made in duplicates for each antibiotic by replacing the inoculum with molecular biology grade water. A growth (positive) control was made in duplicates for each bacteria isolate to replace the antibiotic solution by molecular biology grade water. The 96-well plates were incubated at 37°C for 16 to 20 hours (Wiegand et al., 2008). After incubation, Biotek Cytation3 Plate Reader with Gen5 Microplate Data Collection & Analysis Software was used to read the OD600 values. The antibiotic concentration at the well with the lowest concentration that fully inhibited the growth of bacteria were determined as the MIC value of the antibiotic to the bacteria strain. If any bacteria growth observed in the sterile control, inhibition of growth observed in the growth control, or outliers of OD600 were observed in the serial dilution of an antibiotic for a given strain, the microdilution experiment of the corresponding bacteria strain would be repeated. Bacteria strains with a MIC greater than the CLSI threshold of a given antibiotic would be considered resistant to the antibiotic (data available upon reasonable request).

### 2.6 *E. coli* sequencing and annotation

In addition to the environmental *E. coli* isolated in this study, we also received three *E. coli* strains isolated from human clinical samples from the healthcare facility located in Town A with no identifiable information available. The high-molecular weight (HMW) *E. coli* genome DNA was extracted by GenFind v3 (Beckman Coulter). The quantity and quality of the DNA extracts were determined by NanoDrop. The HMW DNA extracts were sheared by Megarupter 3 (Diagenode) at speed 40 before PacBio HiFi sequencing library preparation. Inhibitors were removed from the sheared DNA extracts by OneStep PCR Inhibitor Removal Kit (Zymo Research) followed by 1:1 bead cleaning using SMRTbell® cleanup beads (PacBio). PacBio HiFi sequencing libraries were made using the SMRTbell prep kit 3.0 (PacBio). The DNA yields of the libraries were determined by Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit (Invitrogen) and DNA fragment lengths were determined by AATI Fragment Analyzer by Roy J. Carver Biotechnology Center before sequencing. Sequencing was conducted on SMRT Revio cell (PacBio) with 30-hour movie time by Roy J. Carver Biotechnology Center. The sequencing reads were assembled *de novo* by Trycycler using Canu, Raven, and Flye as the assemblers (Kolmogorov et al., 2019; Koren et al., 2017; Vaser & Šikić, 2021; Wick et al., 2021). The *E. coli* genomes were annotated by BV-BRC Genome Annotation service. The integron regions were determined by IntegronFinder 2.0 (Néron et al., 2022).

# 3 Results and Discussion

### 3.1 Integron presence was associated with significantly higher plasmid ARG loads across incompatibility groups, host genera, and plasmid sizes.

Among the 1102 ARG-carrying plasmids collected from PLSDB RefSeq, 419 carried at least one integron-associated structures, including complete integron, In0, or CALIN element, and 683 did not have any above structures identified. The 419 plasmids with integron-associated structures were labeled integron-positive, and the remaining 683 were labeled integron-negative. The number of ARGs carried by integron-positive plasmids were significantly higher than integron-negative plasmids, with medians of 9 and 2, respectively ($p < 0.0001$) (**Fig. 1a**). The distribution of ARG numbers for the two types of plasmids also showed different patterns. The number of ARGs on integron-negative plasmids is skewed at low levels (e.g., # of ARGs ≤ 3 for most plasmids), while the number of ARGs on integron-positive plasmids were more spread out along the Y axis to a higher level (e.g., approximately from 3 to 29).

We further divided the plasmids by different incompatibility groups (IncA, IncF, IncH, IncI, and IncN) and bacterial host genera (*Escherichia*, *Klebsiella*, *Citrobacter*, *Enterobacter*, *Shigella, Salmonella, Acinetobacter*, *Pseudomonas*, and *Enterococcus*). As shown in **Fig 1b**, the numbers of ARGs carried by plasmids in different incompatibility groups were not significantly different ($p = 0.59$). In the same incompatibility groups, the numbers of ARGs on integron-positive plasmids were significantly higher than those on integron-negative plasmids ($p < 0.0001$), except for IncA_C_cgPMLST and IncF_RST that have too few data points. The number of ARGs among different bacterial host genera were distributed differently. However, the similar trend that integron-positive plasmids carried significantly more ARGs than integron-negative plasmids was also observed in six host genera, including *Escherichia* ($p < 0.0001$), *Klebsiella* ($p < 0.0001$), *Citrobacter* ($p < 0.0001$), *Enterobacter* ($p < 0.01$), *Salmonella* ($p < 0.0001$), and *Acinetobacter* ($p < 0.0001$) (**Fig. 1c**). No plasmids hosted by *Enterococcus* were found carrying any integron-associated structures. The above findings suggest that the higher number of ARGs carried by integron-positive plasmids were not restricted or dominated by a certain incompatibility group or bacterial host genus.

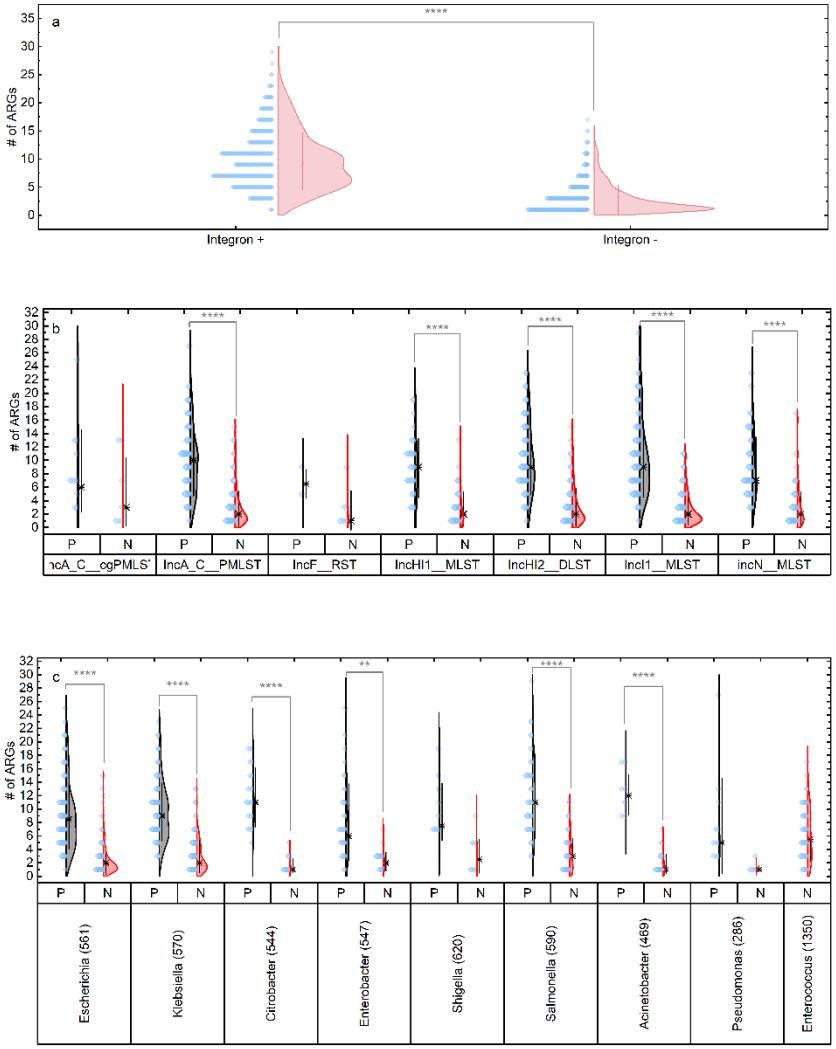


**Fig 1.** The number of ARGs on integron-positive plasmids is significantly higher than on integron-negative plasmids (a). The significant increase in ARG numbers for integron-positive plasmids (black) compared to integron-negative plasmids (red) can also be observed across incompatibility groups (b) and bacterial genera (c). *Enterococcus* does not contain any integron-positive plasmids.

Intuitively, larger plasmids have more spaces to carry more genes. We, then, investigated the effect of plasmid size on ARG numbers and integron presence. As expected, with plasmid size increasing, the maximum number of ARGs on plasmids also increased (**Fig. 2a**). However, from approximately $2\times10^3$ bp to $2\times10^6$ bp, the minimum number of ARGs on ARG-carrying plasmids was constantly one. From approximately $2\times10^3$ bp to $4\times10^5$ bp, the number of ARGs on plasmids gradually spread out from the same minimum number to a wider range, instead of moving upward along the Y axis (**Fig. 2a**). We, then, categorized the plasmids by size into tertiles using the 33rd and 67th percentile cutoffs (P33 = 58125 bp, P67 = 118444 bp). In each tertile, the integron-positive plasmids contained significantly more ARGs than integron-negative plasmids ($p < 0.0001$) (**Fig. 2b**). In addition, the median numbers of ARGs carried by integron-positive plasmids increased from 5 to 7 to

11 as the plasmid size increased, but those median numbers on integron-negative plasmids were 2, 2, and 3, respectively. The association between integron presence and increased ARG counts became more pronounced on larger plasmids, while integron-negative plasmids showed only limited increases in ARG load. This finding suggested the presence of integrons was linked to the additional expansion of ARG-carrying capacity on large plasmids.

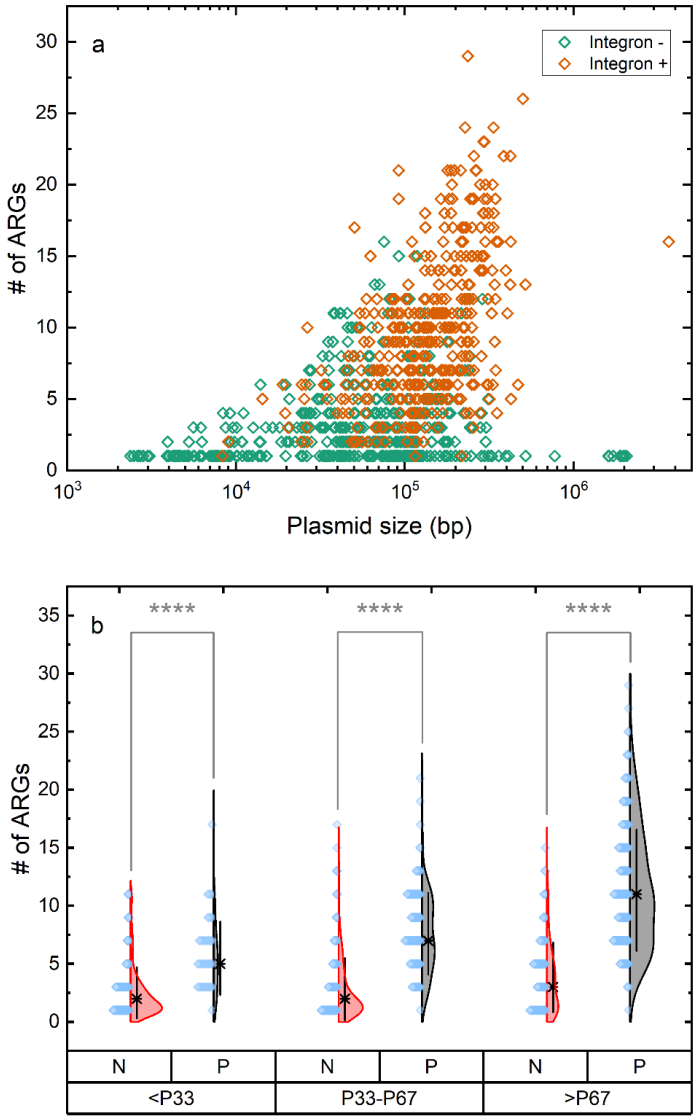


**Fig 2.** Increased plasmid size leads to increased maximum number of ARGs a plasmid can carry (a). However, integron-positive plasmids contain significantly more ARGs than integron-negative plasmids in all size ranges (b). The asterisks mean the median values of the number of ARGs in each group (P33 = 58125 bp, P67 = 118444 bp).

### 3.2 Additional ARGs on integron-positive plasmids were not restricted to integron cassette arrays.

We subsequently asked whether the increased number of ARGs on integron-positive plasmids were solely due to the cassette arrays that integrons carried. We classified the ARGs carried by each plasmid into two categories: inside integron (at least 1 bp overlap with the integron region) and outside integron (no sequence overlaps with the integron region). Because *sul1* and *qacEdelta1* were often considered as conserved structures of part of Class 1 integrons but the sequences of these two genes could not be counted into integron regions based on the algorithm of IntegronFinder 2.0, we further made a highly conservative assumption that all plasmids had two ARGs misclassified into the outside-integron group. We therefore increased the number of ARGs on integron-negative plasmids by two before conducting statistical test to compare the number of ARGs outside integron regions on integron-positive plasmids and the number of ARGs carried by integron-negative plasmids. The statistical test results showed that the number of ARGs outside of integron regions on integron-positive plasmids were significantly higher than the number of ARGs carried by integron-negative plasmids with the two gene misclassification assumption, with medians of 6 and 4, respectively ($p < 0.0001$) (**Fig. 3**). This finding suggests that the increased number of ARGs on integron-positive plasmids was not only due to the ARGs carried by integron cassette arrays, but also other means.

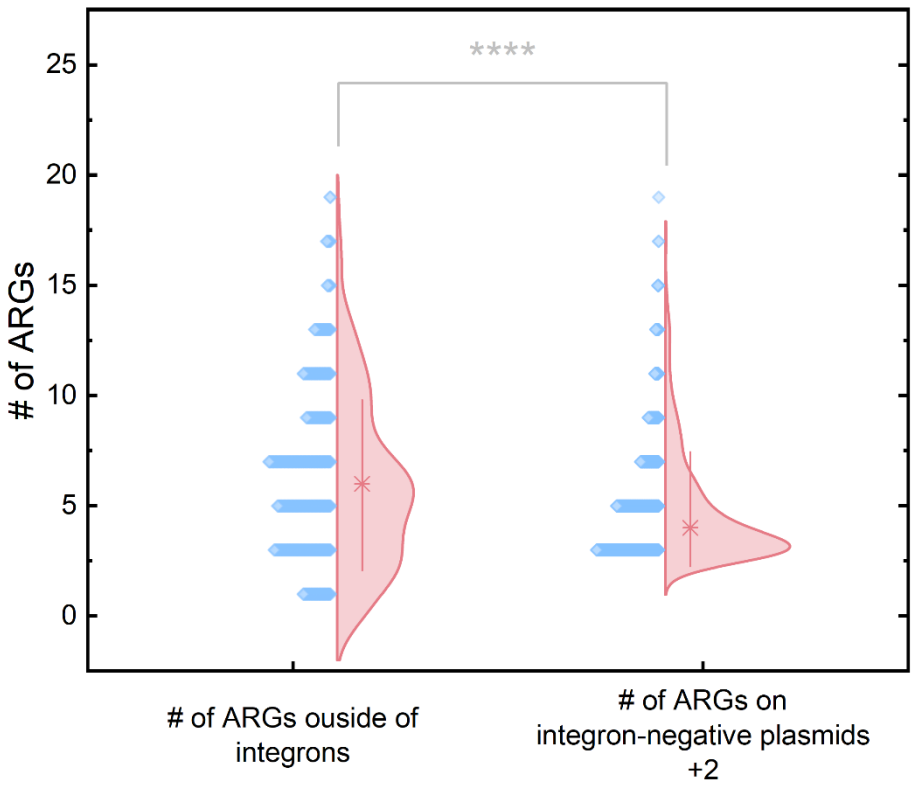


**Fig 3.** The number of ARGs outside of the integron-associated structures are significantly higher than the number of total ARGs on integron-negative plasmids, even with the highly reserved assumption that all integrons contain two ARGs *sul1* and *qacEdelta1* that are excluded from the integron regions. The asterisks mean the median values of the number of ARGs in each group.

### 3.3 Different classes of ARGs show distinct distribution patterns inside versus outside integron regions.

We further asked whether the same ARG classes were found inside or outside the integron regions on integron-positive plasmids. Aminoglycoside resistance genes were the most dominant ARGs both inside and outside integrons, in agreement with previous studies (Corno et al., 2023; Hipólito et al., 2022, 2023). Other ARGs showed different preferences. For example, ARGs conferring resistance to beta-lactam, tetracycline, macrolide, and quinolone tend to distribute outside integron regions, while ARGs conferring resistance to trimethoprim, quaternary ammonium, and rifamycin tend to distribute inside integron regions (**Fig. 4**). ARGs conferring sulfonamide and phenicol resistance did not show distinct preferences inside versus outside. These distributions of the ARGs conferring resistance to different antibiotic classes suggested the difference in the underlying mobilizing mechanisms of these ARGs.

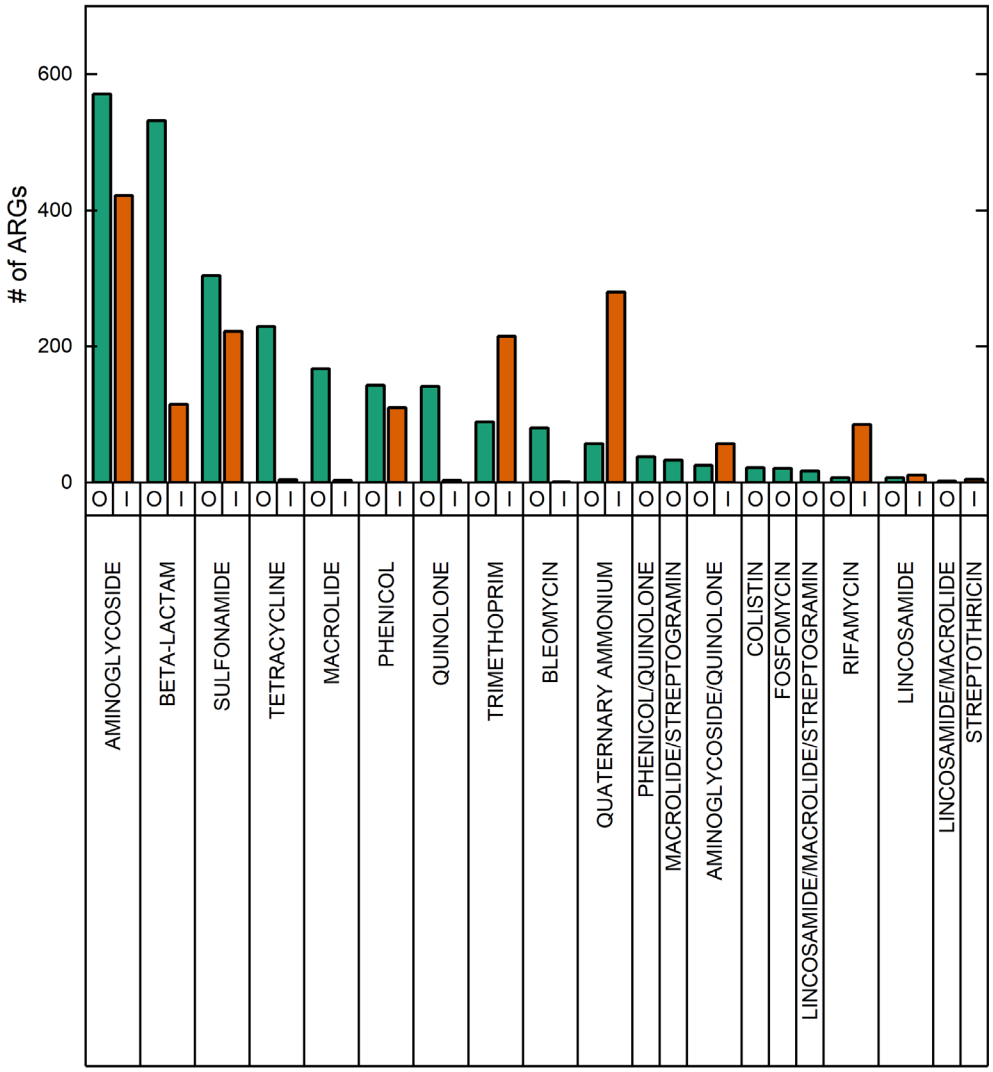


**Fig 4.** The classes of ARGs distributed inside (I) or outside (O) of integrons on integron-positive plasmids.

To understand whether the classes of ARGs distribute differently on similar plasmids with or without integron regions, we analyzed a small lineage of plasmids in depth (n = 65, pairwise ANI > 99.5%) with both integron-positive and integron-negative members. The heatmap in **Fig. 5** shows the distribution of ARGs conferring resistance to different antibiotic classes on each plasmid. Aminoglycoside resistance genes were still the most abundant on integron-positive plasmids, but these genes were rarely found on integron-negative plasmids, suggesting the aminoglycoside resistance genes distributed outside the integron regions may be related to the aminoglycoside resistance genes inside the integron regions. Beta-lactam resistance genes were found on both integron-positive and integron-negative plasmids, suggesting the presence of many beta-lactam resistance genes did not depend on the presence of integrons. Tetracycline resistance genes were not found in any integron regions within this plasmid lineage, suggesting the independency between tetracycline resistance genes and integrons. In addition, ARGs conferring resistance to macrolide and phenicol/quinolone were only identified outside integron regions on integron-positive plasmids, rather than inside integron regions on integron-positive plasmids or integron-negative plasmids. Such correlation may suggest that the presence of integron was linked to the presence of these two ARG classes, but these two ARG classes were not inserted into plasmids by integrons. The presence and absence of these two ARG classes did not fully overlap, suggesting these two classes of ARGs were inserted into this plasmid lineage by a few times, instead of one single event before or after integron insertion. The varied distributions of different ARG classes on integron-positive and integron-negative plasmids in a highly similar lineage suggested a mixed mechanism between the presence of integrons and the presence of multiple ARGs on plasmids.

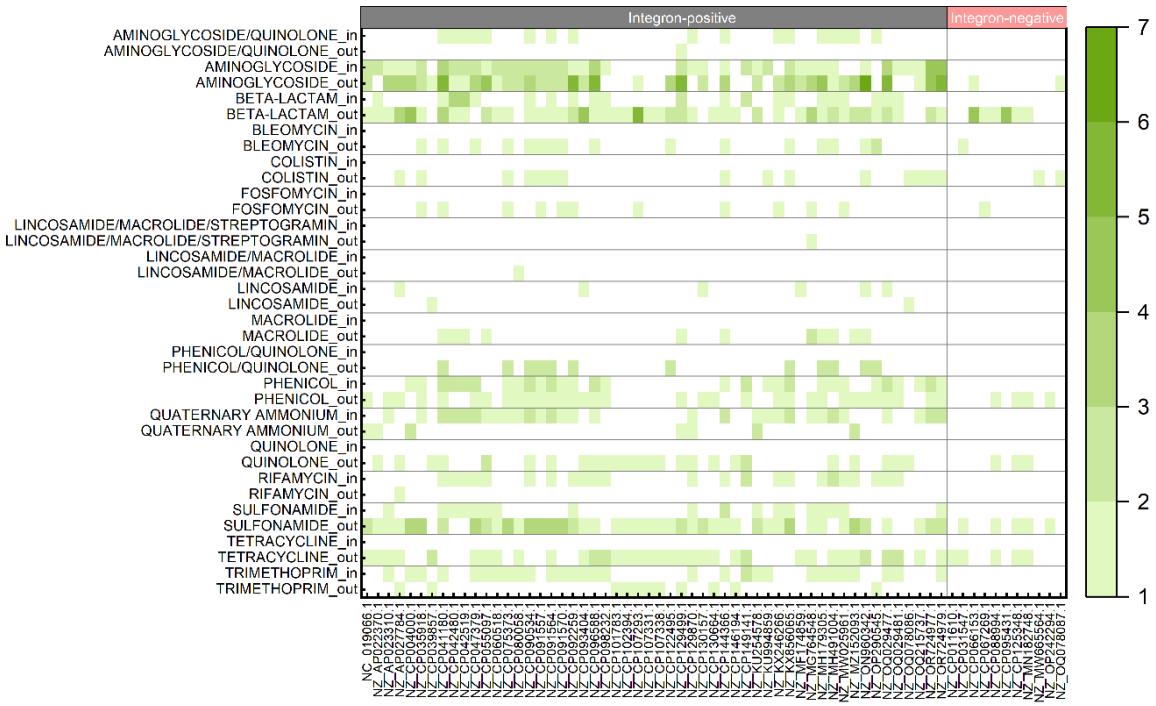


**Fig 5.** The heatmap of the number of ARGs in different ARG classes inside and outside of integrons on integron-positive and integron-negative plasmids in the same lineage (pairwise ANI > 99.5%).

### 3.4 Low-ARG integron-negative plasmids co-exist with high-ARG integron-positive plasmids in the same wastewater sampling sites.

Based on the analysis of the plasmid sequences retrieved from the database, we have shown evidence of the presence of integron was strongly linked to increased number of ARGs on plasmids, and the classes of the ARGs inside and outside integron regions were different. An alternative explanation is that integron-carrying plasmids were co-selected with multi-ARG plasmids under the same antibiotic selection pressure. To reject this alternative explanation, we analyzed plasmids hosted by *E. coli* isolated from different environments, including human community-level raw sewage from two adjacent towns (Town A and Town B), anonymized human clinical samples with no identifiable information available from a hospital in Town A, and wastewater from a pork processing plant in Town B (**Fig. 6a**). We obtained 32 ARG-carrying plasmids among these isolates.

Like the plasmids collected from the database, integron-positive plasmids carried significantly more ARGs than integron-negative plasmids in the environmental samples collected in this study, with medians of 9 and 1, respectively ($p < 0.0001$) (**Fig. 6b**). In addition, the number of integron-positive and integron-negative plasmids were distributed differently among the samples collected from different sources. More integron-positive plasmids were collected from the pork plant, while more integron-negative plasmids were collected from the human community-level raw sewage samples (**Fig. 6c**). We only obtained three *E. coli* isolates from deidentified human samples collected from a hospital (referred as clinical samples), and only one integron-positive plasmid in these *E. coli* isolates carried ARGs. Animal-related and healthcare-related environments were often considered with higher antibiotic selection pressure than human living environments, because antibiotics are regularly used in farms and healthcare facilities, instead of human daily lives (He et al., 2020). The higher proportion of integron-positive plasmids in pork processing plant and clinical samples suggested that *E. coli* isolates with integron-positive plasmids might have better fitness under high antibiotic selection pressure. We further investigated the number of ARGs on integron-positive and integron-negative plasmids collected from Town B wastewater and the pork processing plant, because these two sites contained high enough numbers of plasmids for statistical test. Both sites showed significantly higher numbers of ARGs on integron-positive plasmids than integron-negative plasmids ($p < 0.05$) (**Fig. 6d**). The loci of ARGs and integron regions of the 11 integron-positive plasmids collected in this study can be found in **Fig. S1**, with many ARGs located outside of integron regions. The co-existence of integron-positive and integron-negative plasmids in the same sampling sites suggested that though high antibiotic selection pressure might lead to the selection of more abundant integron-positive multi-ARG plasmids, the numbers of ARGs on integron-negative plasmids in the same environment were still difficult to increase to a level comparable to integron-positive plasmids.

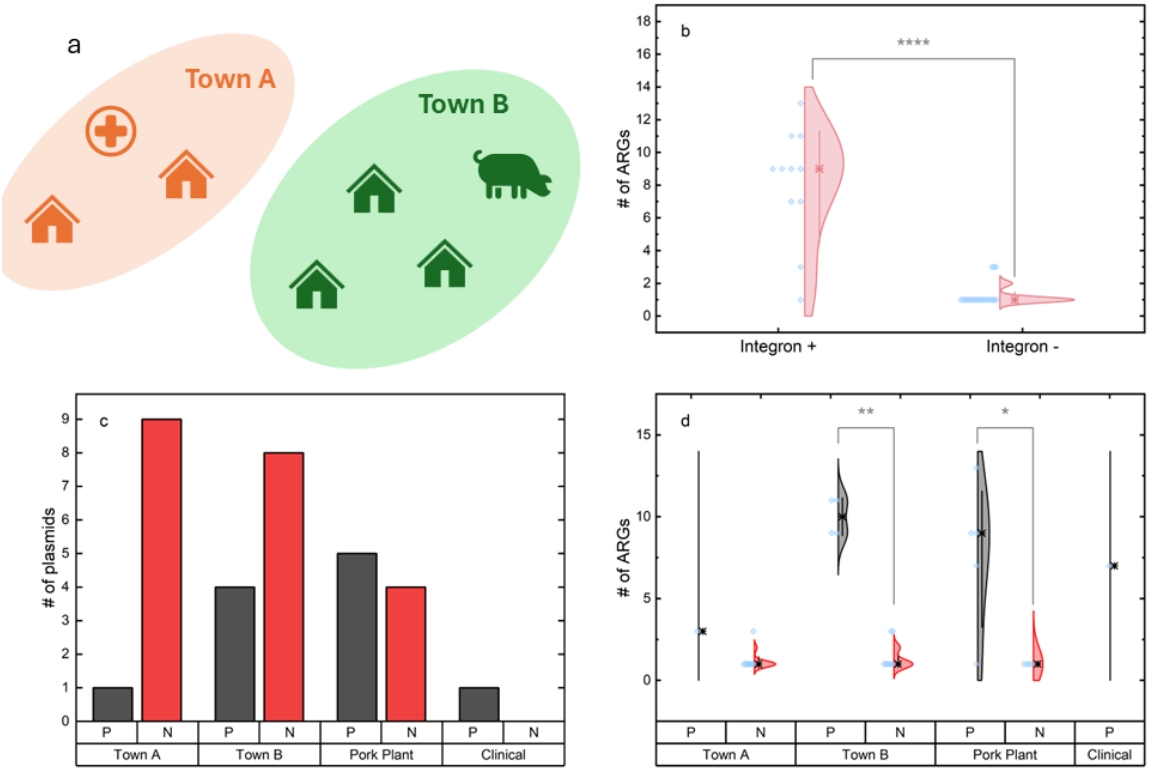


**Fig 6.** The number of ARGs, integron-positive plasmids, and integron-negative plasmids in environmental E. coli samples collected in this study. a) The sampling sites in Town A and Town B. Town A sampling sites included two human communities and a hospital, while Town B sampling sites included three human communities and a pork plant. b) The number of ARGs on integron-positive plasmids was significantly higher than the number of ARGs on integron-negative plasmids in the *E. coli* strains collected in this study. c) The number of integron-positive and integron-negative ARG-carrying plasmids collected from each sampling site. d) For Town B and pork plant, the number of ARGs on integron-positive plasmids were significantly greater than those on integron-negative plasmids. Town A and clinical samples had too few ARG-carrying plasmids to do statistical test.

### 3.5 Exploration of potential explanations for ARG accumulation on integron-positive plasmids

After proving integron-positive plasmids were not co-selected with multi-ARG plasmids, we investigated alternative explanations of the integron-correlated ARG accumulation using the database data. We asked whether the accumulation of ARGs on integron-positive plasmids were due to the fragmentation of a larger collapsed integron or independent mosaic events, as illustrated in **Fig 7a**. When the cassette arrays of a large integron collapsed into fragments, at least one CALIN element should be detected, because the CALIN element is defined as sequences with *attC* sequences but no *intI* gene (Cury et al., 2016). In contrast, if other ARGs were inserted into the plasmid by independent mosaic events, the *intI*-containing sequences including complete integrons and In0 should be more dominant. Previous studies defined mosaic plasmids as plasmids receiving genetic elements from different sources (Pesesky et al., 2019). We compared the number of CALIN elements

and the number of complete integrons plus In0 for plasmids carrying one integron and plasmids carrying multiple integrons. We found that CALIN elements were rarely detected on these plasmids (**Fig. 7b**). Seventy of 98 plasmids with two or more integrons region lack CALIN elements, and 298 of 321 integron regions on plasmids with single integron were not CALIN elements. The majority of integron-associated structures contained the *intI* sequence. Because *intI* is located exclusively at the 5′ end of an intact integron, the observation that the majority of integron-associated structures in our dataset contain *intI* rather than CALIN elements indicates that most integrons remain structurally intact instead of being fragmented on ARG-carrying integron-positive plasmids.

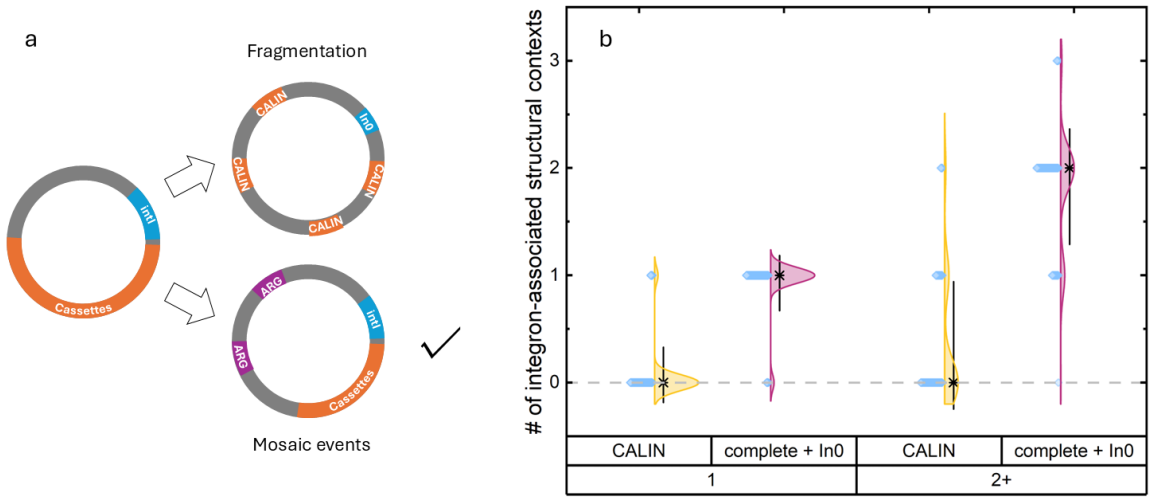


**Fig 7.** The number of CALIN elements and intI-containing integrons on different integron-positive plasmids can be used to infer the potential mechanism of how integron-positive obtain multiple ARGs. a) The conceptual figure of two different ways for a plasmid to get multiple ARGs after receiving an integron. Fragmentation from a large integron will generate separated CALIN, but mosaic events can introduce ARGs without generating CALIN. b) The numbers of complete integrons or In0 were significantly greater than the number of CALIN on integron-positive plasmids, suggesting integrons were not frequently fragmented.

IS26 is regarded as an important mechanism contributing to the mobility of integron-associated sequences (Dawes et al., 2010; He et al., 2015; Zhao et al., 2018). In addition, IS26 was also considered contributing to the rearrangement of a wide variety of ARG classes (Oliva et al., 2018; Tang et al., 2024; Y. Wang & Dagan, 2024). Here, we determined the correlation between the presence of integrons and presence of IS26 insertion sequences on the 1102 ARG-carrying plasmids. By setting the BLAST identity cutoff to 80% and the length coverage cutoff to ≥500 bp, we classified the plasmids to IS26-positive and IS26-negative. As expected, IS26-positive plasmids were more dominant in integron-positive plasmids than integron-negative plasmids (**Fig. 8a**). We then evaluated the mixed effects of IS26 insertion sequences and integrons on the number of ARGs on plasmids. The number of ARGs were significantly different across all four groups, with increased median from 1 to 2 to 6 to 10 for integron-negative IS26-negative, integron-negative IS26-positive, integron-positive IS26-negative, and integron-positive IS26-positive (**Fig. 8b**), suggesting that the presence of IS26 insertion sequences was linked to the further increased number of ARGs

on integron-positive plasmids. These findings indicate that plasmids with IS26-like sequences but currently lacking integrons may form genomic contexts that are more permissive to future integron insertion and ARG acquisition and the presence of IS26 insertion sequences partially contributed to the ARG number increase on integron-positive plasmids.

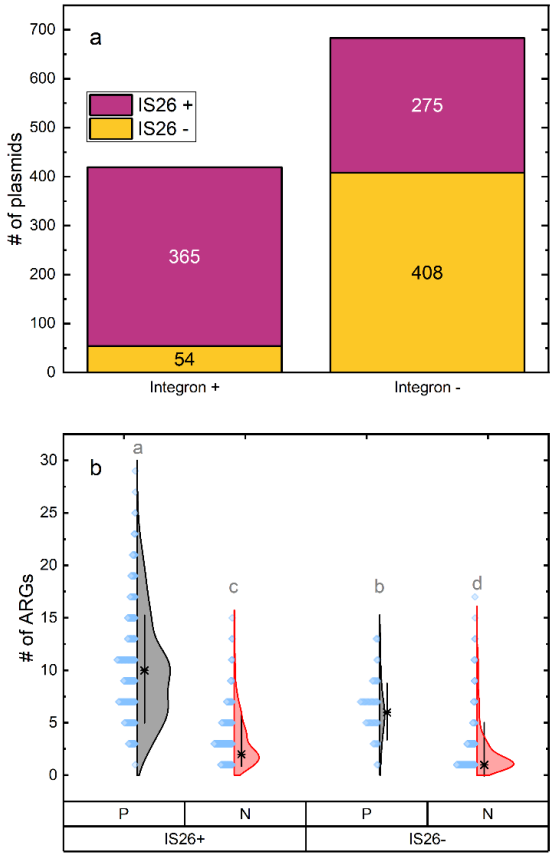


**Fig 8.** a) More integron-positive plasmids tend to contain IS26 insertion sequences than integron-negative plasmids. b) The comparison of the number of ARGs on the plasmids with or without IS26 or integrons present.

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