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Flavomycin inhibits plasmid-mediated conjugative transfer of antibiotic resistance genes by disrupting energy metabolism and pilus assembly

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ABSTRACT

The rapid global dissemination of multidrug-resistant (MDR) bacteria, primarily driven by horizontal gene transfer through conjugative plasmids, poses a significant challenge to modern medicine. Conjugation enables the efficient spread of antibiotic resistance genes across bacterial populations, severely compromising the efficacy of existing therapies. This study examined the inhibitory potential of flavomycin against plasmid-mediated transmission of clinically relevant resistance genes and elucidated the underlying molecular mechanisms. Results showed that flavomycin markedly reduced the conjugative transfer of plasmids carrying bla_{CTX-M}, bla_{NDM}, and *mcr-1* genes in a dose-dependent manner, decreasing conjugation frequencies by approximately 14- to 100-fold. Mechanistic analysis indicated that inhibition of plasmid transfer resulted from intracellular depletion of ATP and L-arginine, both essential for the energy-dependent conjugation process. Transcriptomic analyses revealed broad suppression of genes involved in energy metabolism, while supplementation with exogenous L-arginine restored conjugation frequencies. Additionally, flavomycin down-regulated the expression of mating pair formation (MPF) genes and disrupted pilus biogenesis, as confirmed by scanning electron microscopy. These findings identify flavomycin as a potent inhibitor of horizontal gene transfer, acting through disruption of bacterial energy metabolism and impairment of pilus assembly, and highlight its potential as a promising strategy to limit the propagation of MDR bacteria.

Keywords: Antibiotic resistance genes; Plasmids; Flavomycin; Dissemination control

INTRODUCTION

The escalating global spread of *Enterobacteriaceae* harboring extended-spectrum β-lactamases (ESBLs) and carbapenemases (CREs) represents a major threat to public health and antimicrobial stewardship. These pathogens are classified as high-priority by the World Health Organization (WHO) (Tacconelli et al., 2018) due to their extensive resistance profiles and limited therapeutic options. ESBLs efficiently hydrolyze oxyimino-β-lactams such as cefotaxime, ceftazidime, and aztreonam at rates reaching at least 10% that of benzylpenicillin, and their activity is strongly inhibited by clavulanate (Castanheira et al., 2021). In contrast, CREs exhibit broad-spectrum β-lactam hydrolytic activity, including against carbapenems, and their widespread transmission across various hosts and environments exacerbates their public health burden (He et al., 2023). Among the limited effective therapeutic options for treating these resistant infections, colistin remains one of the last-line agents for managing infections caused by ESBL- and CRE-producing strains. However, the emergence of plasmid-encoded mobilized colistin resistance genes, including *mcr-1* and its variants, now spanning over 60 countries, has further compromised treatment efficacy and intensified international concern (Liu et al., 2016, 2024).

The rapid dissemination of bla_{CTX-M}, bla_{NDM}, and *mcr-1* genes is predominantly driven by horizontal gene transfer, with conjugation serving as the most efficient and pervasive mechanism (Liu et al., 2020). Conjugative plasmids mediate the inter- and intraspecies transmission of these resistance determinants, accelerating the spread of multidrug-resistant (MDR) bacteria across clinical and environmental reservoirs

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(Partridge et al., 2018). Plasmid types such as IncI2, IncX4, IncHI2, IncP, IncX3, and IncFII have been identified as the dominant vectors for the worldwide mobilization of bla_{CTX-M}, bla_{NDM}, and *mcr-1* (Liu et al., 2023b, 2024; Partridge et al., 2018). Mechanistic studies have revealed that IncI2 plasmids encoding *mcr-1* utilize ProQ/FinO systems to modulate the trade-off between resistance gene expression and bacterial fitness, ensuring both maintenance and dissemination (Yang et al., 2021). Similarly, IncX4 plasmids bearing *mcr-1* rely on the transcriptional regulator PixR to promote high-frequency conjugation, contributing to their successful propagation (Yi et al., 2022). The growing prevalence and adaptability of these resistance genes present a formidable challenge to infection control, underscoring the urgent need for innovative strategies targeting the molecular machinery of conjugative transfer to curb the global spread of MDR pathogens (Naghavi et al., 2024).

Given the scarcity of effective antibiotics against MDR strains (Getino & de da Cruz, 2018; Ghosh et al., 2019), disrupting horizontal gene transfer, particularly conjugation, has emerged as a promising strategy to limit the dissemination of antimicrobial resistance (Graf et al., 2019; Wang et al., 2019a; Wu et al., 2022). A range of conjugation inhibitors (COINs), including unsaturated fatty acids, perfluorooctanoic acid, docosahexaenoic acid, biochar, and synthetic peptidomimetics, and dihydroartemisinin (Wang et al., 2023), have demonstrated varying degrees of efficacy in blocking plasmid-mediated gene transfer (Liu et al., 2023a; Palencia-Gándara et al., 2021; Vrancianu et al., 2020; Wu et al., 2022; Zhou et al., 2023). However, the molecular mechanisms by which most COINs exert their effects remain poorly defined, and few have been tested against clinically relevant resistance plasmids, underscoring the need to identify and characterize new inhibitors such as flavomycin.

Flavomycin—also known as flavophospholipol or moenomycin—is a phosphorylated polysaccharide antibiotic originally used as a feed additive to promote growth in livestock and control Gram-positive infections (Chen et al., 2019; Ostash & Walker, 2010). It acts by selectively inhibiting glycosyltransferases, essential for peptidoglycan biosynthesis, without exhibiting cross-resistance with β-lactam antibiotics that also target cell wall assembly (Ostash et al., 2010; Vanderwel & Ishiguro, 1984). Although previous studies have reported that flavomycin reduces the prevalence of resistance genes in gut microbiota and suppresses the conjugation of certain plasmids (Braná et al., 1973; George & Fagerberg, 1984; Poole et al., 2006), its effects on the conjugation of clinically relevant plasmids harboring important resistance genes have not been thoroughly explored. Moreover, the molecular basis by which it interferes with conjugative transfer has not been elucidated. This study systematically investigated the impact of flavomycin on the conjugation of resistance plasmids and explored the potential mechanisms underlying its inhibitory activity.

MATERIALS AND METHODS

Bacterial strains

Nine *Escherichia coli* strains isolated from food-producing animals in China were selected as donors for conjugation assays (Supplementary Table S1). Strain identity was confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonik GmbH, Germany). Polymerase chain reaction (PCR)-based replicon typing (PBRT) was performed on each isolate and its corresponding transconjugant or transformant carrying a single plasmid, following previously established protocols (Carattoli et al., 2005). The donor panel included three isolates harboring the colistin resistance gene *mcr-1* on epidemic IncI2, IncX4, and IncP plasmids; three strains harboring the extended-spectrum β-lactamase (ESBL) gene bla_{CTX-M} on IncFII (F33) and IncFII (F2) plasmids; and three strains harboring the carbapenemase-encoding gene bla_{NDM} on IncFII (K2) and IncX3 plasmids. A streptomycin-resistant *E. coli* C600 derivative was used as the recipient strain in the conjugation assays.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was evaluated using the minimum inhibitory concentration (MIC) assay, following the Clinical and Laboratory Standards Institute guidelines (CLSI 2023). Briefly, single colonies were inoculated into 3 mL of Mueller-Hinton broth (MHB) and incubated overnight with shaking (180 r/min) at 37°C. The following day, 2 μL of the overnight culture was diluted 1:1 000 in 3 mL of MHB and incubated with shaking at 37°C for 4 h. A 100 μL aliquot of the resulting suspension (10^6 CFU/mL) was added to each well of a 96-well microtiter plate containing serial 2-fold dilutions of antimicrobial agents in 100 μL of MHB. Plates were incubated without shaking at 37°C for 18 h. MICs were defined as the lowest concentration at which visible bacterial growth was inhibited. Streptomycin, colistin, cefotaxime, and flavomycin were tested, with *E. coli* ATCC 25922 used as the control strain (Supplementary Table S1).

Evaluation of flavomycin effects on bacterial growth

Donor and recipient strains at the exponential phase were diluted in MH broth to approximately 10^6 CFU/mL. Donor and recipient growth kinetics were assessed by inoculation in 10 mL of fresh Luria-Bertani (LB) broth containing 0, 4, 8 or 16 mg/L flavomycin and incubated at 37°C with shaking. Optical density at 600 nm (OD_{600}) was recorded every hour for 12 h. All experiments were conducted in triplicate.

Conjugation assays

Conjugation experiments were performed following previously described protocols (Riedl et al., 2000), with slight modifications. On day 1, donor strains were cultured separately in LB broth containing 0, 8, or 16 mg/L flavomycin and incubated overnight at 37°C (pre-cultures). The recipient strain was cultured in LB broth without flavomycin. On day 2, overnight cultures of both donor and recipient strains were diluted 1:100 into fresh LB broth and incubated at 37°C with shaking for 4 h. Equal volumes of donor and recipient cultures were then combined and co-cultured statically overnight in the presence of 0, 2, 4, 8, or 16 mg/L flavomycin. On day 3, 10-fold serial dilutions of the co-cultures were plated on LB agar supplemented with selective antibiotics to quantify transconjugants. Plates containing 2 mg/L colistin and 3 000 mg/L streptomycin were used to select *mcr-1*-positive transconjugants, while plates with 2 mg/L cefotaxime and 3 000 mg/L streptomycin were employed to select for bla_{NDM} or bla_{CTX-M} transconjugants. To assess the influence of L-arginine on plasmid conjugation frequency, 1 mmol/L L-arginine was added to the co-cultures on day 2, and all subsequent steps were performed as described above. All conjugation assays were conducted using at least three independent biological replicates.

Conjugation frequency (CF) was calculated by dividing the

number of transconjugants by the number of *E. coli* C600 recipients. Relative conjugation frequency (relative CF%) was defined as the percentage of CF in the flavomycin-treated group compared to the control group, providing a quantitative measure of flavomycin-mediated inhibition of plasmid transfer (Getino et al., 2015, 2016).

RNA extraction, RNA sequencing (RNA-seq), and bioinformatic analysis

Overnight cultures of *E. coli* SHP16 were grown in LB broth and then diluted 1:100 into fresh LB supplemented with either 0 or 16 mg/L flavomycin (n=3). After 4 h of incubation, cells were harvested by centrifugation at 12 200 ×*g* for 3 min at 4°C, immediately resuspended in RNAprotect Bacteria Reagent (Qiagen, Germany), and centrifuged under the same conditions for 10 min. Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Germany) in accordance with the manufacturer's protocols. RNA integrity and concentration were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and residual genomic DNA was eliminated using RNase-free DNase I (Qiagen, Germany). Subsequently, RNA samples were processed for sequencing on the Illumina HiSeq System (Caporaso et al., 2012), and data analysis was conducted as described previously (Liao et al., 2013). Transcriptome assembly was carried out using Trinity (Haas et al., 2013), and RNA-seq reads were aligned to the *E. coli* BW25113 reference genome (CP009273.1) using Subread (Liao et al., 2013).

Differential gene expression analysis was performed using the fragments per kilobase of transcript per million mapped reads (FPKM) approach. Genes with a false discovery rate (FDR)-adjusted P<0.05 and a fold change≥2 were considered differentially expressed genes (DEGs). Enrichment analyses of DEGs were further conducted to determine involvement in biological pathways and functions using several bioinformatics resources, including the Database for Annotation, Visualization, and Integrated Discovery (https://david.ncifcrf.gov/) (Huang et al., 2009), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 2000), and BioCyc (Caspi et al., 2018) databases.

Quantification of intracellular ATP

Intracellular ATP levels were measured using an Enhanced ATP Assay Kit (Beyotime, China) according to the manufacturer's instructions. Overnight bacterial cultures were diluted 1:100 in fresh LB medium and incubated at 37°C for 4 h. The bacteria were harvested by centrifugation (3220 ×*g*, 4°C, 10 min) and resuspended in 0.1 mol/L phosphate-buffered saline (PBS, pH 7.4) to achieve an OD_{600} of 0.5. Cultures were then treated with 16 mg/L flavomycin for 1 h, followed by cell lysis using lysozyme (1 mg/mL). Supernatants were collected and ATP concentrations were determined using a Spark multimode microplate reader (Tecan, Austria). All assays were conducted with at least three independent biological replicates.

Quantification of intracellular arginine

Intracellular arginine concentrations were quantified using an Arginine Content Assay Kit (Solarbio, China) following the manufacturer's protocols. Overnight bacterial cultures were diluted 1:100 in fresh LB medium and incubated at 37°C for 4 h with or without flavomycin. Bacteria were harvested by centrifugation (3220 ×*g*, 4°C, 10 min), resuspended in fresh LB to an OD_{600} of 0.5, and treated with the assay reagent. Arginine levels were measured using a Spark multimode microplate reader (Tecan, Austria), with excitation at 488 nm and emission at 525 nm. All experiments were performed in triplicate.

Scanning electron microscopy (SEM)

Bacterial morphology was analyzed by SEM. Overnight cultures of *E. coli* SHP16 were diluted 1:100 in 1 mL of fresh LB medium, with or without 16 mg/L flavomycin, and incubated at 37 °C for 4 h. The samples were then washed twice with PBS, centrifuged at 5 000 r/min for 10 min at 4 °C, and fixed with 2.5% glutaraldehyde at 4°C overnight. After fixation, the samples were washed three times with PBS, post-fixed in 1% osmium tetroxide for 30 min, and washed again with PBS. The cells were dehydrated through a graded ethanol series (30%, 45%, 60%, 75%, 85%, 95%, and 100%), with each step lasting 10 min, followed by two 10-min washes in 100% ethanol. After drying, the bacterial cells were imaged using a Thermo Fisher Version 460 scanning electron microscope (USA).

Statistical analysis

All statistical analyses were conducted utilizing GraphPad Prism v.8.0.2. Differences between two groups were assessed using unpaired t-tests, with results reported as mean±standard deviation (SD). A P-value <0.05 was considered statistically significant. The significant differences between treatment samples and the control are indicated with *: P<0.05, **: P<0.01 and ***: P<0.001.

RESULTS

MIC profiles and effects of flavomycin on bacterial growth

The recipient strain *E. coli* C600 exhibited high-level resistance to both flavomycin and streptomycin (>128 mg/L) (Supplementary Table S1). Among the donor strains, those carrying *mcr-1* (SHP16 carrying the pHNSHP16 plasmid, SHP23 carrying the pHNSHP23 plasmid, and GD6F1 carrying the pHNGD6F1 plasmid) were resistant to flavomycin and colistin. Donor strains harboring bla_{CTX} (FKD228 carrying the pHNFKD228 plasmid, FKD593 carrying the pHNFKD593 plasmid, and FKD565 carrying the pHNFKD565 plasmid) also showed resistance to flavomycin and cefotaxime. Similarly, donor strains carrying bla_{NDM} (AHM6C70 carrying the pHNAHM6C70 plasmid, AHM7C60 carrying the pHNAHM7C60 plasmid, and SDX5C138 carrying the pHNSD138-1 plasmid) were resistant to flavomycin and cefotaxime (Supplementary Table S1). To assess potential growth inhibition, donor and recipient strains were exposed to flavomycin at 4, 8, or 16 mg/L for 12 h at 37°C. As illustrated in Supplementary Figure S1, none of these concentrations substantially impaired bacterial viability or growth dynamics, suggesting that flavomycin does not exert bacteriostatic or bactericidal effects under these conditions. These concentrations were therefore selected for subsequent conjugation assays.

Flavomycin inhibits conjugative transfer of resistance plasmids

The effect of flavomycin on plasmid-mediated horizontal gene transfer was assessed through conjugation assays using plasmids encoding *mcr-1*, bla_{CTX-M}, or bla_{NDM}. Flavomycin exhibited a dose-dependent inhibition of plasmid conjugation (Figure 1; Supplementary Table S2). At 8 mg/L, conjugation

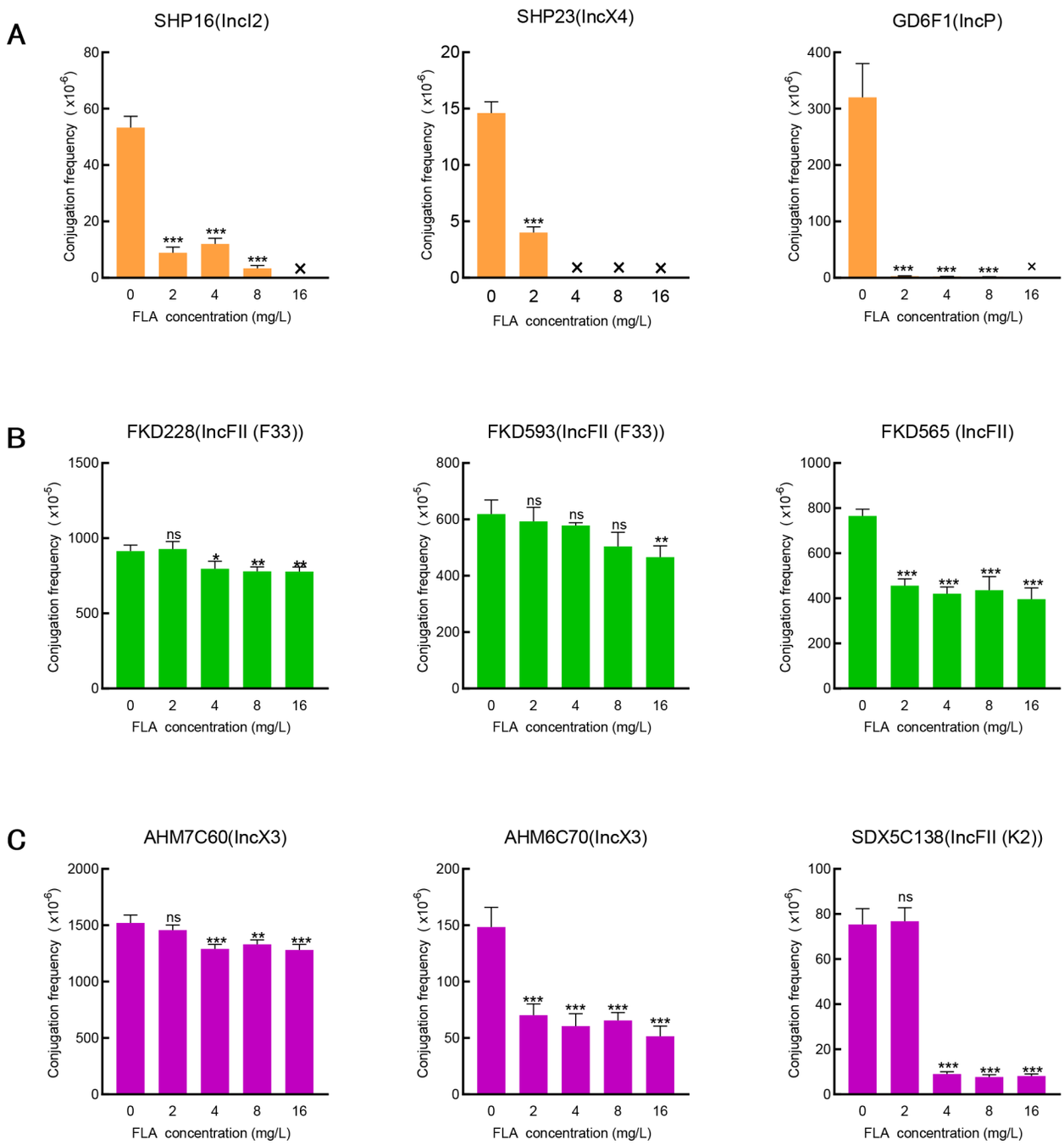

Figure 1 Effects of flavomycin on conjugation frequency without pre-culture

A–C: Changes in conjugative transfer frequency of *mcr-1*-bearing plasmids (A), $bla_{CTX\text{-}M}$-bearing plasmids (B), and bla_{NDM}-bearing plasmids (C). No transconjugants were detected. Significant differences between control and treatment samples are indicated as: ns: Not significant; *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

frequencies of *mcr-1*-bearing plasmids (pHNSHP16, IncI2; pHNSHP23, IncX4; pHNGD6F1, IncP) were reduced by up to 100-fold, with frequencies falling below 1% of untreated controls (Supplementary Table S2). At 16 mg/L, no *mcr-1* transconjugants were detected across replicates (Figure 1; Supplementary Table S2). Similarly, flavomycin significantly inhibited conjugation frequencies of plasmids harboring bla_{CTX} (pHNFKD228, IncFII (F33); pHNFKD593, IncFII (F33); pHNFKD565, IncFII (F2)) and bla_{NDM} (pHNAHM6C70, pHNAHM7C60, IncX3; pHNSD138-1, IncFII (K2)), all exhibiting at least a 10-fold reduction in conjugation frequency, with levels falling below 10% of control values (Supplementary Table S2).

To further investigate the temporal dynamics of inhibition, donor strains were pre-incubated with flavomycin prior to conjugation. Pre-incubation at 8 or 16 mg/L further reduced plasmid transfer efficiency, particularly for *mcr-1*-positive plasmids (pHNSHP16, IncI2; pHNSHP23, IncX4; pHNGD6F1, IncP), where conjugation frequencies dropped below the CF detection limit of 10^{-7} at 8 mg/L (Supplementary Figure S2; Supplementary Table S3). These findings suggest that prolonged flavomycin exposure may exert a cumulative inhibitory effect, potentially altering donor cell physiology in a manner that disrupts the conjugative apparatus or impairs

plasmid mobilization.

Flavomycin disrupts energy metabolism required for conjugative transfer

To determine whether flavomycin interferes with the cellular energy supply necessary for plasmid conjugation, intracellular ATP concentrations were quantified in *E. coli* cells following flavomycin exposure. As shown in Figure 2A, treatment with flavomycin resulted in a significant decrease in ATP levels. This reduction in ATP is consistent with the inhibition of conjugation, as lower ATP availability likely hampers the energy-driven processes involved in conjugative transfer.

Transcriptomic profiling further revealed broad suppression of genes associated with core energy metabolism. Notably, genes encoding succinate dehydrogenase (*sdhABCD*) were significantly down-regulated following flavomycin treatment (Figure 2B). In addition, genes related to the tricarboxylic acid (TCA) cycle, such as *aceB*, *aceK*, *fumA*, and *fumC*, as well as those involved in glycerol utilization (*glpD*, *glpF*, *glpK*, and *lldP*), were down-regulated following exposure (Figure 2B). These transcriptional changes are consistent with the observed reduction in ATP levels (Figure 2A) and pronounced decline in conjugation frequency (Figure 1), indicating that flavomycin disrupts energy-generating pathways essential for conjugative transfer.

Flavomycin-induced L-arginine depletion restricts plasmid conjugation

Further analysis of RNA-seq data revealed that flavomycin exposure triggered significant up-regulation of genes involved in L-arginine biosynthesis and catabolism (*argABEGHIR*), as well as genes encoding components of the L-arginine transport system (*artI*, *artJ*, *artM*, *artP*, and *artQ*) (Figure 3A, B). These observations suggest that flavomycin induces intracellular depletion of L-arginine, which is known to play a crucial role in cellular metabolism and protein synthesis (Zhang et al., 2024). Consistently, genes associated with glutamine uptake and biosynthesis (*glnP*, *gltB*, *gltD*, and *gltP*) (Figure 3A) were also up-regulated, likely reflecting a compensatory response to arginine depletion, consistent with previous findings (Zhang et al., 2024). Direct quantification confirmed a significant reduction in intracellular L-arginine levels following flavomycin exposure (Figure 3C), suggesting that depletion of this amino acid contributes to the observed inhibition of plasmid conjugation. To assess the functional relevance of this depletion, exogenous L-arginine was added to the conjugation system. As shown in Figure 3D, the addition of L-arginine enhanced conjugation frequency in the presence of flavomycin, suggesting that intracellular L-arginine depletion is a key mechanistic contributor to the suppression of plasmid transfer by flavomycin.

Flavomycin suppresses conjugation-associated gene expression and impairs pilus formation

To assess the impact of flavomycin on pilus formation, transcriptional profiles of mating pair formation (MPF) genes associated with IncI2-type plasmids were examined. As shown in Figure 4A, flavomycin treatment led to significant down-regulation of genes involved in Type IV secretion system biogenesis, including *traG* and *traF*, which mediate DNA processing and mating pair stabilization, as well as *traEJKL*, which encode core components of the pilus and secretion channel (Guglielmini et al., 2014). Additionally, genes within the *pil* locus (*pilS*, *pilR*, *pilP*, *pilQ*, and *pilU*), which enhance conjugation efficiency in IncI2 plasmids (Bradley, 1984; Neil et al., 2021), were also significantly suppressed following flavomycin treatment (Figure 4A).

The impact of flavomycin on pilus morphology was further confirmed by SEM. As expected, pili were clearly observed on *E. coli* cells not exposed to flavomycin (Figure 4B), while pili formation was completely abrogated in cells treated with flavomycin (Figure 4C). These findings suggest that flavomycin disrupts pilus formation, thereby impairing the conjugative machinery necessary for plasmid transfer.

DISCUSSION

Horizontal gene transfer via plasmid-mediated conjugation is a principal mechanism driving the dissemination of antibiotic resistance genes among bacteria (Partridge et al., 2018). Although previous studies have indicated that flavomycin can inhibit conjugation in a dose-dependent manner (Braná et al., 1973; George & Fagerberg, 1984; Poole et al., 2006), its effects on clinically relevant plasmids harboring important resistance genes remain insufficiently characterized. This study systematically evaluated the impact of flavomycin on the conjugative transfer of plasmids encoding key resistance determinants and explored its underlying mechanisms of

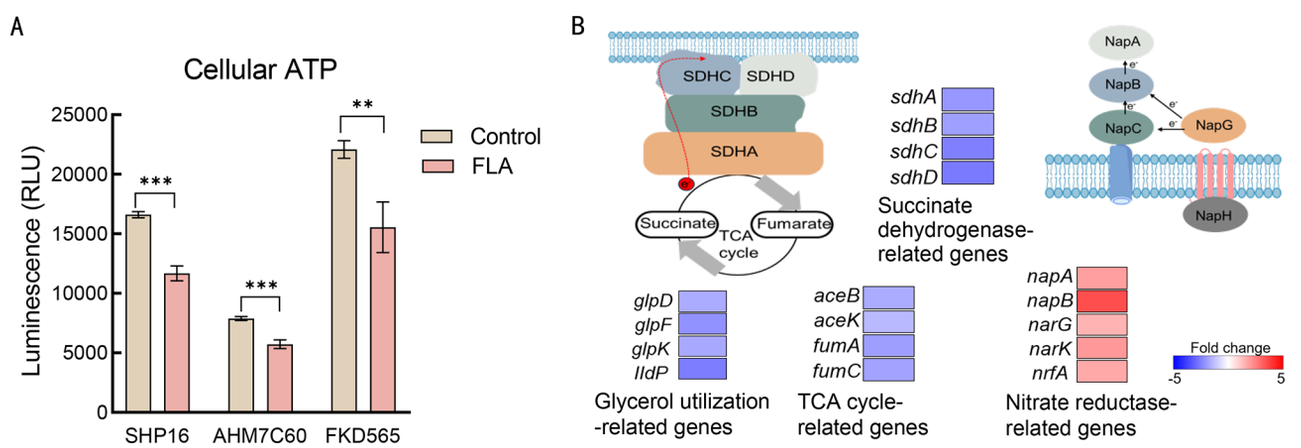

Figure 2 Flavomycin reduces the energy supply required for conjugation

A: Relative intracellular ATP levels following flavomycin treatment. B: Transcriptomic changes in energy metabolism-related genes in *E. coli* exposed to flavomycin, with color gradient from blue (down-regulated) to red (up-regulated). Significant differences between control and treatment samples are indicated as: ns: Not significant; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$.

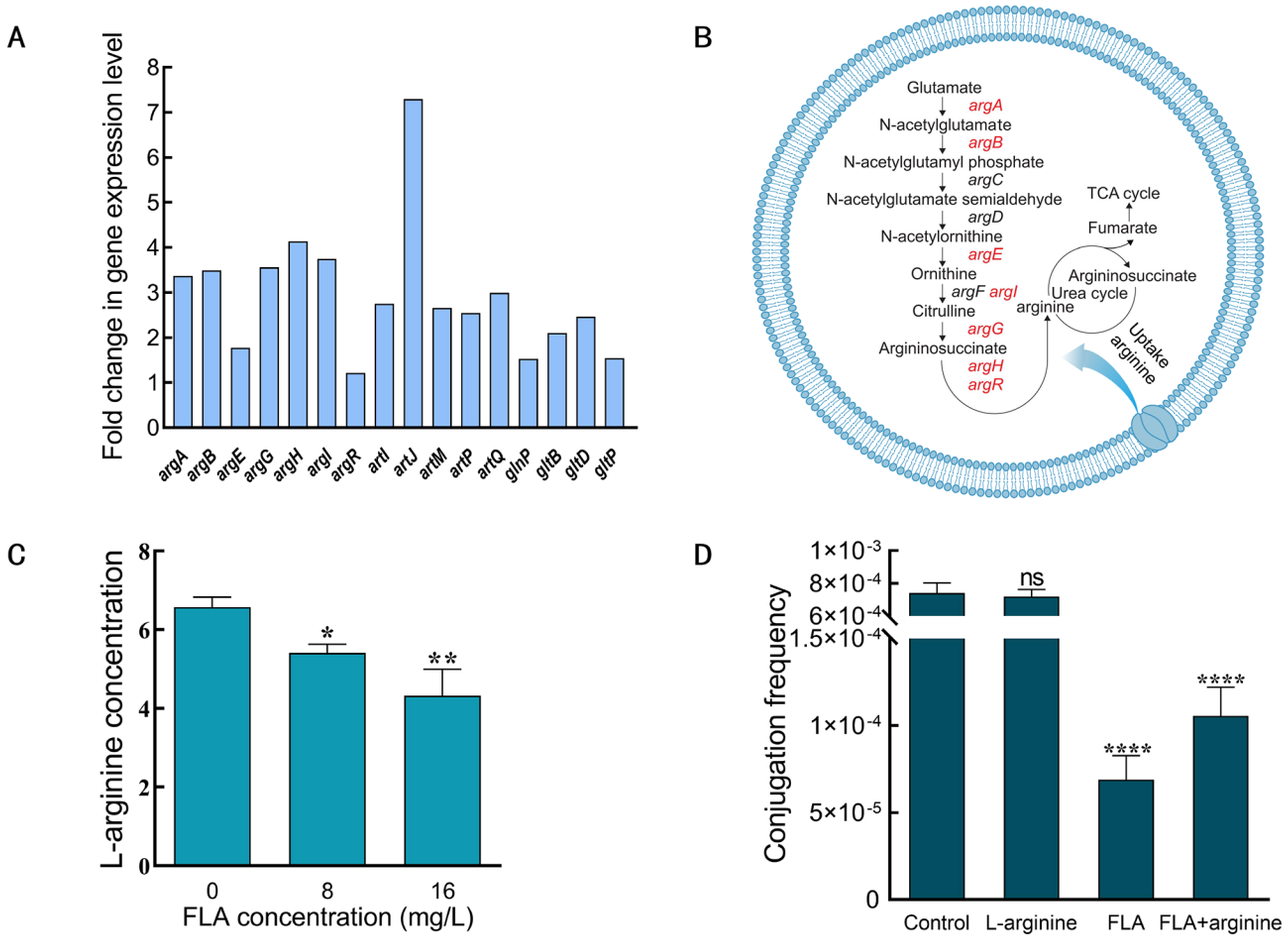

Figure 3 Flavomycin-induced depletion of intracellular L-arginine suppresses plasmid conjugation

A: Transcriptional changes in L-arginine-related genes in *E. coli* following flavomycin treatment. B: Schematic illustrating L-arginine biosynthesis, transport, and catabolism pathways. C: Changes in intracellular L-arginine levels in response to different flavomycin concentrations. D: Conjugation frequency following treatment with 8 mg/L flavomycin, with or without 1 mmol/L exogenous L-arginine supplementation. Significant differences between control and treatment samples are indicated as: ns: Not significant; *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

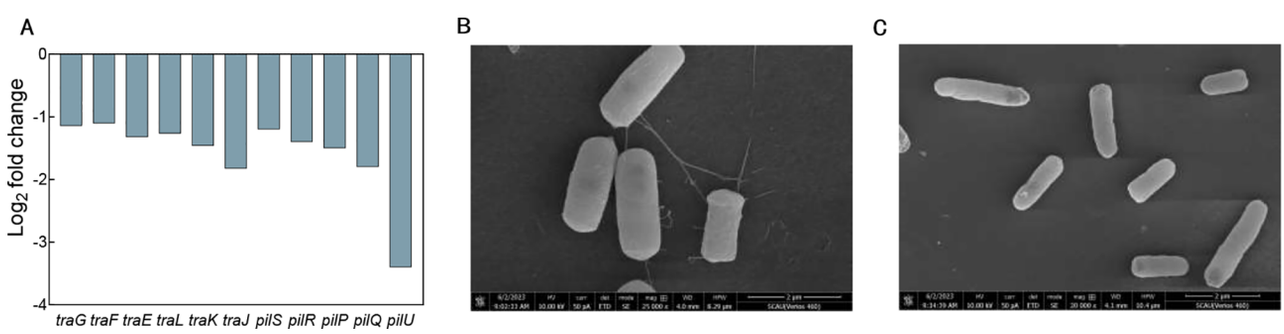

Figure 4 Flavomycin suppresses mRNA expression of conjugation-related genes and pilus formation

A: Transcriptional changes in genes associated with conjugation. B: Scanning electron microscopy (SEM) image showing piliation state in vehicle-treated cells. C: SEM image showing piliation state in flavomycin-treated cells.

action.

Consistent with earlier findings, flavomycin did not exhibit bactericidal activity against Gram-negative bacteria such as *E. coli* (Kudo et al., 2019; Poole et al., 2006). Its antibacterial activity is restricted to Gram-positive bacteria, where it targets glycosyltransferases involved in peptidoglycan biosynthesis (Ostash & Walker, 2010). In Gram-negative species, the outer membrane serves as a barrier that blocks entry to the cell wall synthesis machinery, preventing flavomycin from exerting antimicrobial effects (Heaslet et al., 2009; Ostash & Walker, 2010). Despite its lack of bactericidal activity, flavomycin strongly inhibited conjugative plasmid transfer. Previous reports described only modest reductions in conjugation frequency in *E. coli*, typically ranging from 1.4- to 3-fold (Kudo et al., 2019). In contrast, the present study demonstrated markedly stronger inhibition, with conjugation frequencies reduced by 14- to 100-fold (corresponding to transfer efficiencies below 1% to 7.37%; Supplementary Tables S2, S3). This enhanced suppression may reflect differences in bacterial hosts, plasmid types, or assay conditions, all of which are known to influence conjugation efficiency (San Millan et al., 2014; Yano et al., 2016). Previous research has

suggested that the inhibitory activity of flavomycin may be plasmid-type specific (George & Fagerberg, 1984). However, other studies have suggested that at higher concentrations, flavomycin broadly inhibits conjugation regardless of plasmid type, with specificity only emerging under low-transfer conditions (Kudo et al., 2019). In the present study, flavomycin produced stronger inhibition of *mcr-1*-encoding plasmids (IncI2, IncX4, and IncP) compared to those carrying bla_{CTX} (IncFII) or bla_{NDM} (IncX3 and IncFII). This discrepancy may be partially explained by the selective pressure applied during conjugation assays. In particular, *mcr-1*-positive transconjugants were selected on LB agar supplemented with both colistin and streptomycin, which may have amplified the inhibitory effect of flavomycin through synergy with colistin (Huang et al., 2024). Compared with other reported conjugation inhibitors (Casu et al., 2016; Fernandez-Lopez et al., 2005; Shaffer et al., 2016), flavomycin inhibited plasmid transfer across a wider range of incompatibility groups. This broad inhibitory profile highlights its potential as a promising agent for constraining horizontal gene transfer among MDR bacteria, a process that plays a central role in the global escalation of antimicrobial resistance.

Plasmid conjugation is an energy-intensive process, requiring ATP for both DNA transfer and the assembly of conjugative machinery, including pilus biogenesis (Cabezón et al., 2023; Chen et al., 2005; Huang et al., 2019; Yu et al., 2020). Although the decline in ATP levels following flavomycin treatment did not markedly affect bacterial viability (Supplementary Figure S1), intracellular ATP may have fallen below the threshold required to sustain efficient conjugation (Jewett et al., 2009), contributing to the observed decrease in transfer frequency (Figure 1). Transcriptomic analysis further revealed significant down-regulation in the expression of genes encoding succinate dehydrogenase (*sdhABCD*), which are involved in the electron transport chain (ETC) and ATP generation (Adolph et al., 2024; Mills et al., 2016; Wu et al., 2022). These findings suggest that flavomycin disrupts the electron transfer process, further reducing ATP production. In response to this energy stress, genes involved in anaerobic respiration (*napA*, *napB*, *narG*, *narK*, and *nrfA*) were up-regulated, likely reflecting a compensatory shift toward nitrate and fumarate reductase pathways (Sparacino-Watkins et al., 2014). While these regulatory changes may help sustain cellular viability by enhancing alternative pathways for ATP production (Wang et al., 2019b), they appear insufficient to restore the energy balance necessary for effective plasmid transfer.

Flavomycin also inhibited plasmid transfer through depletion of intracellular L-arginine, a finding consistent with previous studies showing that reduced L-arginine availability impairs conjugation efficiency (Liu et al., 2023a). L-arginine plays a critical role in cellular metabolism, and its deficiency can disrupt the urea cycle, leading to altered metabolic transitions in the TCA cycle and impaired oxidative metabolism (Shambaugh III, 1977; Zhang et al., 2024). Therefore, flavomycin-induced depletion of L-arginine may reflect its metabolic conversion to ammonia, a process that elevates intracellular pH, buffers against acid stress, and helps maintain pH homeostasis (Xiong et al., 2016). While this adaptive shift may support cell survival under stress, it likely interferes with energy metabolism, thereby impairing the efficiency of plasmid conjugation. The precise molecular pathways linking arginine catabolism, pH regulation, and conjugation remain to be fully elucidated.

In addition to metabolic disruption, flavomycin impaired conjugation by suppressing the expression of MPF genes that encode components of the Type IV secretion system and pilus assembly machinery (Waksman, 2019). In this study, transcriptional profiling revealed significant down-regulation of genes required for pilus biogenesis, corresponding with a complete loss of pili observed by electron microscopy. These findings indicate that flavomycin disrupts the physical structure required for mating pair formation, thereby inhibiting plasmid transfer through both metabolic interference and direct suppression of the conjugative apparatus.

CONCLUSION

Flavomycin effectively inhibits the conjugative transfer of clinically relevant plasmids harboring antibiotic resistance genes, interfering with multiple cellular processes essential for horizontal gene transfer. This study identified the mechanisms underlying the inhibitory effects of flavomycin on plasmid conjugation, including disruption of energy production through ATP and L-arginine depletion and suppression of pilus formation required for MPF. These combined effects impair the conjugation machinery at both metabolic and structural levels. Given the growing global threat of MDR pathogens, flavomycin—already approved as a livestock feed additive—offers considerable potential as a targeted strategy to restrict the spread of resistance genes and a promising candidate in the fight against antimicrobial resistance.

DATA AVAILABILITY

All sequencing data were deposited in the National Center for Biotechnology Information (NCBI) database (BioProjectID PRJNA1274612), Genome Sequence Archive (GSA) database (https://ngdc.cncb.ac.cn/gsa/) (accession number PRJCA041443), and Science Data Bank (doi: 10.57760/sciencedb.j00139.00237).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

J.H.L. and Y.Y.L. designed the study. M.L., Y.J.L., C.Y.L., R.R.Q., J.H.L., J.K.C., H.Y.Y., X.L.L., and Y.H. carried out the experiments. Y.Y.L. and J.H.L. drafted and revised the manuscript. All authors read and approved the final version of the manuscript.

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