Spring 6-2025

Exploring Natural Competence of *Escherichia coli* Through Biofilm Formation

Thulni Liyanaarachchi
Portland State University

Exploring Natural Competence of *Escherichia coli* Through Biofilm Formation

by

Thulni Liyanaarachchi

An undergraduate honors thesis submitted in partial fulfillment of the

requirements for the degree of

Bachelor of Science

in

University Honors

and

Biology

Thesis Advisor

Dr. Justin Courcelle

Portland State University

2025

Table of Contents

Acknowledgement

I would like to express my deepest gratitude to those who have assisted me through this process: Professor Justin Courcelle, my thesis advisor, for giving me the opportunity to complete research within his lab and guidance; Dr. Charmain Courcelle for sharing her expertise and insights on my research topic and experiments; and my lab members for their support and assistance with the research process.

Abstract

Escherichia coli is a species not known for natural competence; however, specific growth conditions can induce the process in this bacterium due to the presence of homologous competence genes. These conditions could be met with biofilm formation through an active growth phase in the two-layer architecture. Therefore, my thesis examined whether natural competence is exhibited in *E. coli* during biofilm formation as determined by the genetic transfer of ampicillin and chloramphenicol-resistance genes between two separate strains. The experiments were broken into three aims to determine the morphology of the biofilm in inter-strain co-cultures and cell-DNA mixes, the effect of media supplementation on antibiotic resistance marker transfer, and the role of biofilm formation on genetic exchange. I found distinct "pink, dry, and rough" (pdar) morphology for inter-strain co-cultures and cell-DNA mixes when at least one of the strains was able to synthesize cellulose for biofilm formation. Co-cultures and mixes with strains unable to synthesize cellulose only displayed irregular concentric circles of growth around the periphery. The types of growth medium influenced the morphology and growth rate of biofilms with co-cultures grown on $CaCl_2$ -supplemented Congo Red medium displaying distinct pdar and wrinkled morphology and faster growth on double selective plates (ampicillin/chloramphenicol). Mixing purified genomic DNA from a strain containing one antibiotic resistance marker with an intact bacterial strain with the opposite marker resulted in a higher occurrence of doubly resistant bacterial colonies compared to co-cultures made through mixing of two intact bacterial strains. However, whole genome sequencing of a colony isolate from ampicillin and chloramphenicol double selection showed the presence of only one of the two antibiotic resistance markers, indicating the transfer of genetic markers might be transient or non-recombinogenic. Further research is required to determine the mechanism for the presentation of double antibiotic resistance.

Introduction

Natural competence is the uptake of exogenous naked DNA for horizontal gene transfer without mobile genetic elements (8). Four steps are required for natural competence and successful transformants to occur: development of cell competence, DNA binding, DNA uptake and processing, and phenotypic expression of newly incorporated genes (2). *Escherichia coli* is not known to have natural competence without artificial induction. However, *E. coli* does have several genes that are homologous to genes of naturally competent bacterial species. Several growth parameters have been shown to induce Gram-negative bacteria to exhibit natural competence including high metabolic activity with appropriate nutrients, Ca^{2+} and Mg^{2+} supplementation, nutrient limitations, pH, and temperature (2,8,11).

Biofilm communities form within an extracellular polymeric substance matrix, which maintains the structure and introduces new functions for the biofilm such as controlled growth rate and metabolic processes (1). Biofilm architecture has been shown to be influenced by both curli fibers and the exopolysaccharide cellulose in this extracellular matrix. (10,19,21). There are two layers in biofilm formation defined as a vegetative growing phase and a stationary phase. The vegetative growing phase is located on the bottom and edge of the biofilm near the nutrient source of the plate, while the stationary phase is located on the top layer of an aggregate colony where cells exhibit starvation due to a nutrient gradient (1,14,15). The proximity between microbial cells within a biofilm also leads to an increase in genetic material exchange with interplay between biofilm formation and horizontal gene transfer. (1). Species with known natural competence such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, have been shown to upregulate competence genes in biofilm growth (14). Given these conditions, biofilms may present an appropriate condition for *E. coli* to exhibit natural competence.

The morphology of biofilms is distinct depending on the types of growth medium and bacterial strains. AR3110 is derived from *E. coli* strain W3110 to contain a stop codon to leucine modification in *bcsQ*, resulting in expression of a full-length BcsQ and cellulose production (14). Congo Red is an amyloid dye which binds to amyloid fibers found in biofilm wrinkles to clearly identify morphology (7). When AR3110 is grown on Congo Red plates, it presents wrinkles called rdar for red, dry, and rough (7,14). The wrinkles are also formed on YESCA agar, which includes yeast extract and casamino acids, and when grown with thioflavin S for dye there is an increase in size and quantity of wrinkles with time (2,14). In contrast, W3110 colonies grow in irregular concentric rings starting on the outer circumference and moving in over time when cultured on Congo Red and YESCA plates (13,14).

Along with biofilm formation, the supplementation of calcium also contributes to the potential natural competence of *E. coli*. Calcium is a known regulatory mediator for bacterial competence and transformation of *E. coli* with exogenous DNA (2). Previous studies found a positive correlation between Ca^{2+} in water and transformation frequency as well as evidence of

natural transformation in *E. coli* grown on agar plates with varying concentrations of calcium, but not LB liquid media (2,13).

If *E. coli* does exhibit gene transfer during biofilm formation, it would suggest a cellular mechanism to uptake exogenous DNA and induce competence that has not been described in this species and would be an alternative mechanism to the known horizontal gene transfer pathways. Such a finding would also expand the purpose of biofilm formation beyond protection and survival in the environment. To determine the impact of biofilm formation on natural competence in *E. coli*, I focused on the distinct colony morphologies of cellulose producing AR3110 and cellulose synthesis-deficient W3110 genetically marked with ampicillin or chloramphenicol resistance markers; the effect of metal supplementation in the growth medium on biofilm formation and genetic transfer through acquisition of antibiotic resistance; and the occurrence of doubly antibiotic-resistant strains using inter-strain co-cultures and cell-DNA mixes.

Methods

Bacterial strains. W3110 and AR3110, a derivative of W3110 containing a mutation in *bcsQ* (stop6leu) (14), were used as parental strains in this study and were a generous gift from L. Cegelski. The *recF*::Tn3 (ampicillin resistant) and *xonA*-HA::cat (chloramphenicol resistant) alleles from SR108 *recF*::Tn3 (HL930,3) and BW25113 *xonA*-HA::cat (CL5278, Courcelle unpublished results), respectively, were transferred utilizing P1 phage into AR3110 and W3110 to produce AR3110 *recF*::Tn3 (CL5575), AR3110 *xonA*-HA::cat (CL5573), W3110 *recF*::Tn3 (CL5576), and W3110 *xonA*-HA::cat (CL5574).

Plate media for macrocolony biofilm formation. YESCA plates (1% Casamino acids, 1.5% agar, and 0.1% yeast extract) were used to induce macrocolony biofilm formation. Where indicated, agar plates were supplemented with Congo Red (40 µg/mL), and 5 mM $CaCl_2$ or 5 mM $MgCl_2$. Antibiotics were supplemented in media to 100 µg/mL ampicillin and 20 µg/mL chloramphenicol.

Genomic DNA purification. Cells from 1.5-mL aliquots of fresh overnight cultures of W3110 *recF*::Tn3 and W3110 *xonA*-HA::cat were pelleted down by centrifugation, resuspended in 500 µL of lysis buffer (2 mg/mL lysozyme, 0.1 mg/mL RNAseA in 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0), and incubated at 37˚C for 45 minutes. Then, 20 µL 20% Sarkoysl and 20-µL 10 mg/mL ProteinaseK were added to each sample and incubation at 37˚C continued for an additional 30 minutes. Following incubation, samples were extracted once with two volumes of a 1:1 mixture of phenol and chloroform and once with two volumes of chloroform before dialysis against TE (2 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0).

Inter-strain co-cultures and cell-DNA mixes. Strains were cultured in salt-free liquid Luria-Bertani (LB) medium overnight. For inter-strain co-cultures, 40-µL aliquots of overnight culture from each strain in a paired co-culture were pre-mixed in an Eppendorf tube. For cell-DNA

mixtures, 50-µL aliquots of overnight culture (~5 x 10^7 cells) and 25-µL purified genomic DNA, equivalent to the amount of DNA from 5 x 10^7 cells, were pre-mixed in an Eppendorf tube. 10-µL aliquots of each mixture were spotted on YESCA plates with or without supplementation as described above. Plates were incubated at room temperature for 5 days before macrocolonies were collected and assessed for morphology and acquisition of antibiotic resistance.

PCR analysis of ampicillin- and chloramphenicol-resistant progeny from inter-strain co-cultures and cell-DNA mixes. The *recF* and *xonA* regions of the genome from ampicillin and chloramphenicol resistant progeny were PCR amplified with primers recFup 5'gaagcggaagagatcctcga and recFdown 5'gacatcaacgtttctcgctc or xonAcheckF 5'cctcaggagtttcaaatagct and xonAcheckR 5'tggcagaaatggtcagcggcg. PCR products were analyzed by agarose gel electrophoresis

Results

Morphology of aggregate colonies arising from inter-strain co-cultures is dependent on growth medium and supplementation.

To observe aggregate colony morphology, I propagated co-cultures of cellulose-proficient and -deficient strains of *E. coli* on the growth medium YESCA, which promotes biofilm formation (7), and compared their macrocolony phenotypes. In order to distinguish between the partner strains used in each co-culture, AR3110 (cellulose producing) and W3110 (cellulose synthesis-deficient) strains were each tagged with either ampicillin- or chloramphenicol-resistance genes using P1 transduction to produce AR3110 *recF*::Tn3 (AR3110 ampR), AR3110 *xonA*-HA::cat (AR3110 camR), W3110 *recF*::Tn3 (W3110 ampR), and W3110 *xonA*-HA::cat (W3110 camR). Overnight cultures of strains were mixed pairwise, plated on YESCA agar plates or YESCA plates that were supplemented with Congo Red, $CaCl_2$ or $MgCl_2$, and incubated at room temperature for five days to allow for the optimal viewing of morphology.

Co-cultures of AR3110 ampR (AR amp) × AR3110 camR (AR cam) produced microcolonies that were large and glossy on YESCA plates with a single radial ridge on the periphery of the biofilm (Figure 1). Macrocolonies from Congo red supplemented-YESCA and YESCA with both Congo red and $MgCl_2$ supplementation were large, deep red, and displayed similar morphology to each other with one distinct ridge growth near the periphery of the biofilm (Figure 1). In contrast, AR amp × AR cam grown on YESA Congo Red and $CaCl_2$ medium produced small, dry, pink aggregate colonies that had rough/wrinkled (pdar) morphology around the periphery (Figure 1).

To determine the effect of cellulose production on biofilm morphology, I compared the phenotypes of co-cultures of AR amp× AR cam, W3110 ampR (W amp) × W3110 camR (W cam), AR amp × W cam, W amp ×AR cam on all four types of media. The exhibition of pdar morphology was dependent on at least one of the co-culture strains being able to produce cellulose (AR3110) (Figure 1). Interestingly, I observed small differences between co-cultures

AR amp × W cam and W amp × AR cam with AR amp × W cam macrocolonies resulting in a uniform pdar morphology on the periphery and some tangled fibers in the middle (Figure 1). When both partners in a co-culture were cellulose deficient (W3110), aggregate colonies appeared shiny with irregular concentric circle growth (Figure 1).

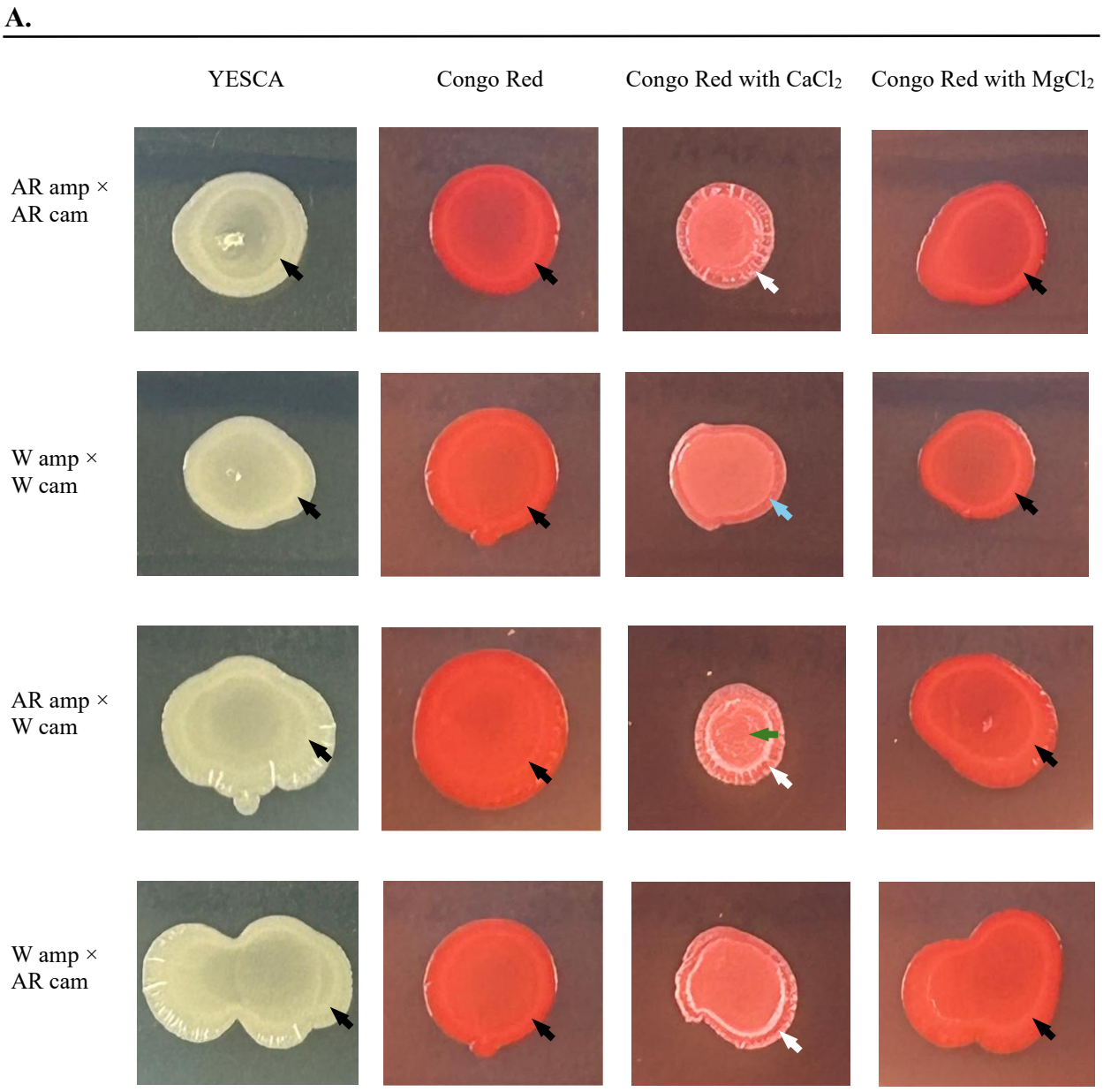


Figure 1. Morphology of aggregate colonies from inter-strain co-cultures is dependent on growth medium and supplementation. Aggregate colonies from the AR 3110 ampR (AR amp) and AR3110 camR (AR cam) co-culture display rdar morphology with Congo Red and calcium chloride supplementation and morphology of aggregate colonies from inter-strain co-cultures display pdar morphology when at least one of the strains is able to synthesize cellulose (AR3110). Representative aggregate colonies from AR amp × AR cam, W3110 ampR (W amp) × W3110

camR (W cam), AR amp × W cam, and W amp × AR cam co-cultures grown on yeast extract-casamino acid (YESCA) agar plates, YESCA supplemented with Congo Red, YESCA with Congo Red and $CaCl_2$, and YESCA with Congo Red and $MgCl_2$, as indicated, after five days of growth at room temperature are shown. Black arrows indicate the radial ridge around the periphery of the biofilm, white arrows indicate the pdar morphology, the green arrow indicates the tangled fibers at the center and the blue arrow indicates the irregular concentric circles of the biofilm.

Morphology of aggregate colonies arising from cell- DNA mixes.

The results above suggested that the ability to produce cellulose and supplementation of the growth medium with calcium affected the morphology of biofilms. Since calcium is a known co-factor for bacterial transformation and cell-mediated contact is known to allow horizontal gene transfer (2), it is possible that the observed differences in morphology were part of the natural competence process allowing DNA binding and uptake. To determine whether colony morphology was affected by the presence of exogenous DNA, I mixed purified genomic DNA (gDNA) from W3110 ampR and W3110 camR strains pairwise with intact AR3110 ampR, AR3110 camR, W3110 ampR, and W3110 camR cells, plated these mixes on YESCA agar plates or YESCA plates that were supplemented with Congo Red, $CaCl_2$ or $MgCl_2$, and incubated at room temperature for five days.

When AR3110 ampR (AR amp) cells were mixed with gDNA W3110 camR (gDNA W cam), the resulting colonies on YESCA medium displayed a set of wrinkles on the left side periphery different from pdar morphology (Figure 2A). AR amp cells x gDNA W cam mixes plated on either YESCA supplemented with Congo red or YESCA supplemented with both Congo red and $MgCl_2$ produced similar deep red colonies with no distinct projections or growth patterns. In comparison, AR amp cells mixed with gDNA W cam produced colonies that exhibited the pdar morphology on the periphery as well as a distinct tangled fiber pattern at the center of the biofilm when grown on YESCA supplemented with Congo Red and $CaCl_2$ (Figure 2A).

The remaining combinations of cell-gDNA mixes were also plated on YESCA agar supplemented with Congo Red and $CaCl_2$. Similar to what I observed with inter-strain co-cultures, only cell-gDNA mixes containing cellulose synthesis-proficient AR3110 cells produced colonies with pdar morphology and center fiber tangles (Figure 2B). When AR amp was the intact strain partner, colony morphology was more confined and uniform wrinkles were observed; however, when AR cam was the intact strain used, the resulting colony morphology was less uniform with one edge extending further out and accumulating more wrinkles. When W3110 ampR (W amp) × gDNA W cam was mixed, colonies contained some tangled fibers at the center of the biofilm; however, when the mixes were switched to W cam × gDNA W amp colonies grew with finger-like projections extending out of the periphery and no fibers at the center.

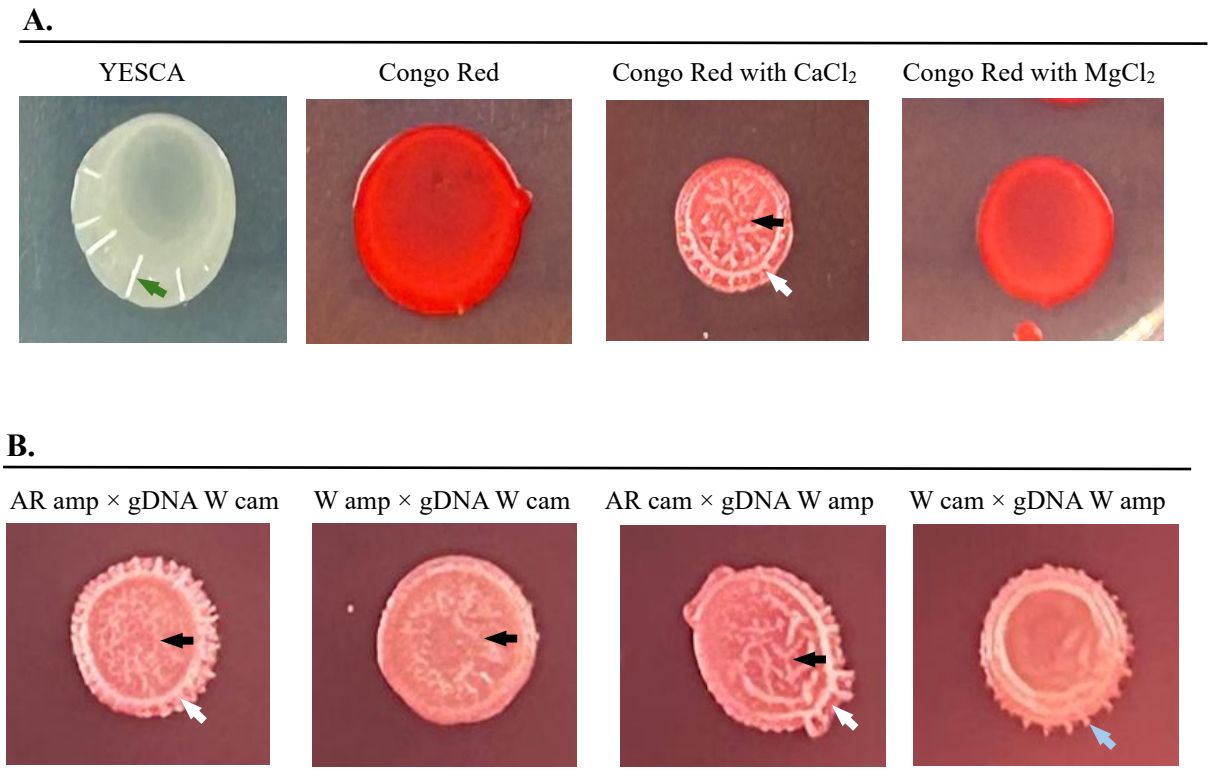


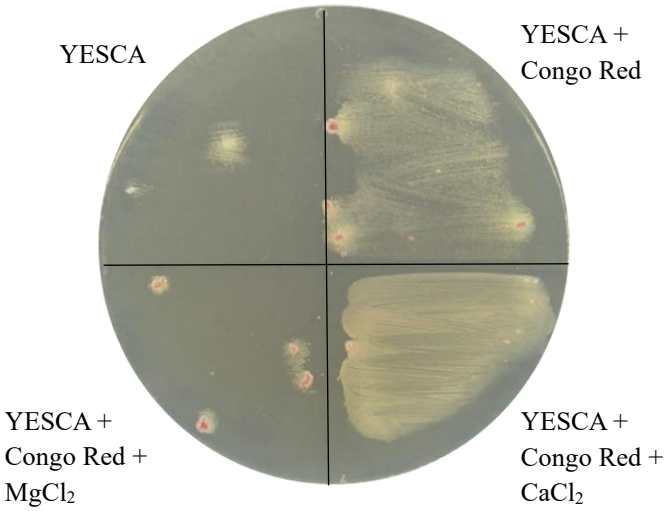
Figure 2. Morphology of aggregate colonies arising from cell- DNA mixes. (A) Aggregate colonies from mixes of AR amp bacterial cultures and W cam purified genomic DNA present pdar morphology on YESCA with Congo Red and calcium chloride supplementation. Representative aggregate colonies from AR amp × gDNA W cam mix grown on YESCA agar plates, YESCA supplemented with Congo Red, YESCA with Congo Red and $CaCl_2$, and YESCA with Congo Red and $MgCl_2$, as indicated, after five days of growth at room temperature are displayed. (B) Morphology of aggregate colonies from cell-DNA mixes present pdar morphology when at least one of the strains is able to synthesize cellulose (AR3110). Representative aggregate colonies from AR amp × gDNA W cam, W amp × gDNA W cam, AR cam × W gDNA amp, and W cam × gDNA W amp mixes on YESCA plates supplemented with Congo Red and $CaCl_2$ after five days of growth at room temperature are shown. White arrows indicate the pdar morphology, black arrows indicate the tangled fibers at the center, the green arrow indicates wrinkles on the left side periphery and the blue arrow indicates the projections of the biofilm.

Progeny from AR amp× AR cam inter-strain co-cultures grown on YESCA medium supplemented with Congo red and calcium chloride grow at an increased rate on ampicillin-chloramphenicol plates.

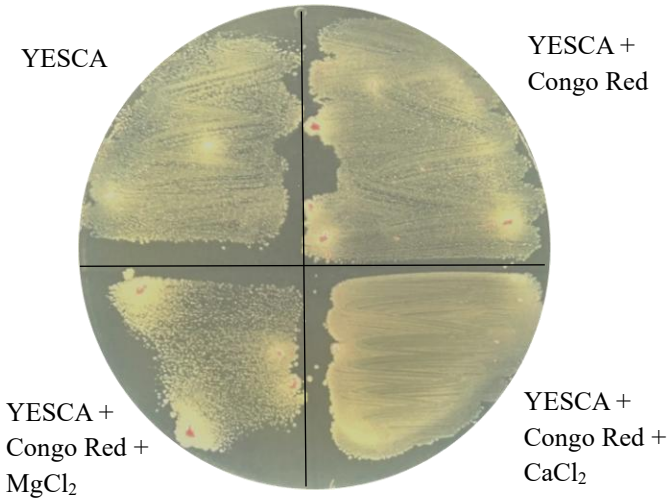
To determine whether biofilm formation facilitates genetic transfer of antibiotic resistance between co-cultures, the aggregate macrocolonies from AR amp× AR cam co-cultures propagated on each type of YESCA and YESCA supplement agar plates were collected and transferred by cotton swab to LB agar plates supplemented with ampicillin and chloramphenicol. After 24 hours of growth in a 37˚C incubator, the biofilms from YESCA with Congo red and

YESCA with Congo red and $CaCl_2$ plates grew as bacterial lawns, with denser growth seen on plates that included $CaCl_2$. Macrocolonies transferred from YESCA agar alone and YESCA supplemented with Congo red and $MgCl_2$ plates produced little to no growth after 24 hours (Figure 3A). However, after 48 hours at 37˚C, biofilm progeny from all four-supplementation media displayed lawn growth (Figure 3B). To determine whether the observed growth represented stable genetic transformation a small amount of all Passage 1 lawns was collected and transferred to fresh LB agar plates containing ampicillin and chloramphenicol and streaked to isolation. If single colonies were obtained on double selective plates in this second serial passage, it would suggest that a cell of one antibiotic resistance phenotype was able to uptake DNA from a cell with the opposite antibiotic resistance. I was unable to isolate single colonies in Passage 2, suggesting there were little to no stable genetic transfected strains for all four-supplementation media (Figure 3C).

A. Serial Passage 1- 24 Hours at 37˚C



B. Serial Passage 1- 48 Hours at 37˚C



C. Serial Passage 2

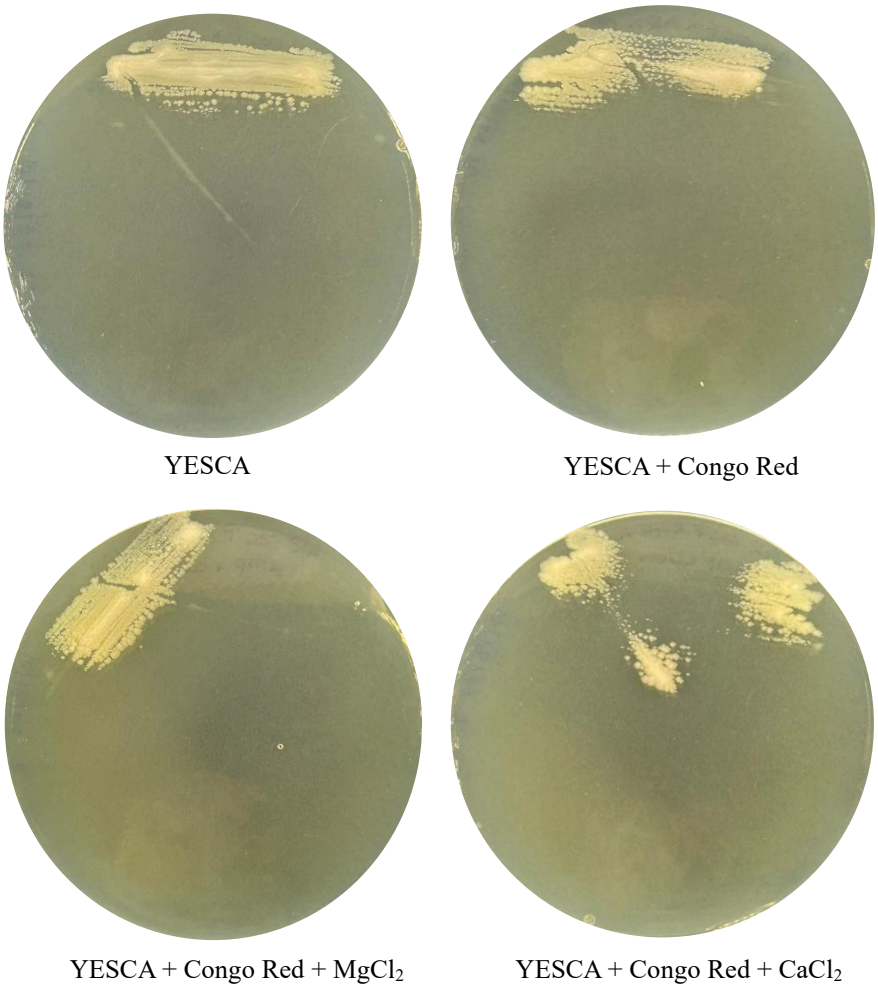


Figure 3. **Progeny from AR amp× AR cam inter-strain co-cultures grown on YESCA medium supplemented with Congo red and calcium chloride grow at an increased rate on ampicillin-chloramphenicol plates**. (A) Outgrowth of biofilm aggregate colonies top left: YESCA, top right: YESCA with Congo Red, bottom left: YESCA with Congo Red and $MgCl_2$, and bottom right: YESCA with Congo Red and $CaCl_2$ after 24 hours and (B) 48 hours of growth at 37˚C on LB agar plates supplemented with ampicillin and chloramphenicol. (C) Bacterial growth on ampicillin-chloramphenicol LB agar plates following serial passage using a small amount of each population labeled YESCA, YESCA supplemented Congo Red, YESCA with Congo Red and $CaCl_2$ and YESCA with Congo Red and $MgCl_2$ from (B) as inoculum.

The ability to synthesize cellulose is required for the development and maintenance of double antibiotic resistance for inter-strain co-cultures

My previous results suggested there was no gene transfer between strains in a biofilm. However, if genetic transfer is a low frequency event, transferring a small amount of a biofilm from one plate to the next might not provide a sensitive enough assay to observe this process nor allow quantitative analysis. Therefore, I modified my experimental procedure by collecting entire macrocolonies from AR amp x AR cam and W amp x W cam co-cultures propagated on YESCA agar plates with Congo red and $CaCl_2$ into LB liquid medium, then performing serial 10-fold dilutions before plating duplicates on LB and LB supplemented with ampicillin and chloramphenicol agar plates. In the absence of antibiotics, progeny from both AR amp x AR cam and W amp x W cam co-cultures showed good viability (~10^9 cfu/ml) and produced uniform colonies (Figure 4A). Of these progeny, ~10^4 cfu/ml were also both ampicillin and chloramphenicol resistant in either AR amp × AR cam or W amp × W cam co-cultures (Figure 4A). Table 1 shows the average frequency of double antibiotic-resistant cells obtained for each co-culture combination at passage 0. A single ampicillin- and chloramphenicol-resistant colony from P0 AR amp × AR cam and W amp × W cam was picked and streaked to isolation on a fresh double selective plate, P1. As shown in Figure 4B, single colonies were obtained for AR amp × AR cam progeny, while W amp × W cam progeny grew as a clump at the site of inoculation, suggesting genetic transfer might have occurred between AR amp and AR cam strains but not between W amp and W cam strains. To confirm this, a single colony from the P1 plate for AR amp × AR cam was picked and serially passaged, P2, on a fresh double selective plate. The resulting single colonies are shown in Figure 4C.

Of the four inter-strain co-cultures, only AR amp × AR cam co-cultures consistently produced doubly antibiotic-resistant colonies that could be serially passaged. AR amp × W cam and W cam × AR amp displayed similar growth to W amp × W cam and did not result in doubly antibiotic-resistant cells in any trials (Table 1).

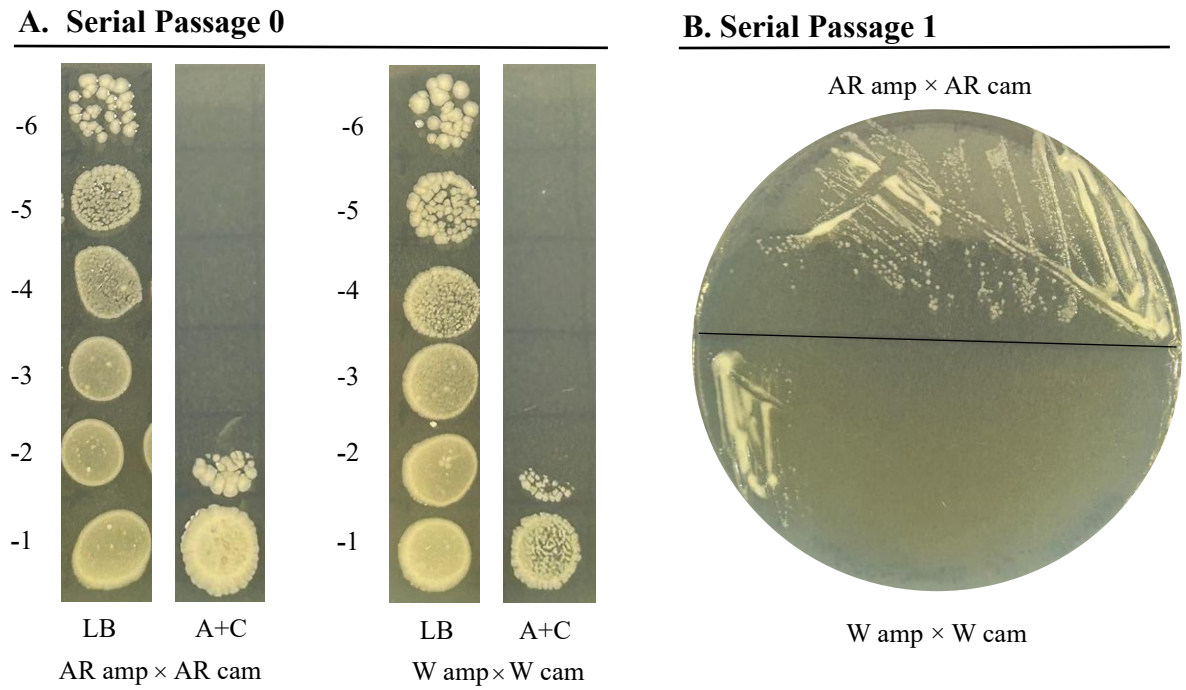


Figure 4. The ability to synthesize cellulose is required for the development and maintenance of double antibiotic resistance for inter-strain co-cultures. (A) Aggregate colonies from inter-strain co-culture biofilm were collected in 1-ml LB medium and 10-fold serially diluted before duplicate 10-µl spots were placed on LB and LB supplemented with ampicillin and chloramphenicol agar plates and incubated for 24 hours at 37˚C (serial passage 0, P0). (B) Single colonies that grew on double antibiotic selective plates from P0 were transferred to fresh ampicillin and chloramphenicol agar plates and streaked to isolation. Serial passage 1 (P1) plate after being incubated for 24 hours at 37˚C is shown. (C) Single colonies from P1 were serially passaged on fresh ampicillin- chloramphenicol agar plates and streaked to isolation (serial passage 2, P2).

Table 1. Frequency of ampicillin-chloramphenicol double resistant colonies from inter-strain co-cultures grown on YESCA with Congo red and $CaCl_2$ agar.

Inter-strain co-cultures	Average number of amp-cam resistant cells per million at passage 0*	First Serial Passage		Second Serial Passage	
		Number of colonies plated	Number fully grown out to isolated colonies**	Number of colonies plated	Number fully grown out to isolated colonies**
AR amp × AR cam	82	8	5	5	2
W amp × W cam	83	4	0	-	-
AR amp × W cam	73	4	0	-	-
W amp × AR cam	23	1	0	-	-

*Average of at least 3 independent experiments shown except for W amp× AR cam co-culture, which was performed once.
** The presence of isolated colonies was assessed after 24 hours of growth at 37˚C.

E. coli cells mixed with purified genomic DNA are able to acquire and maintain double antibiotic resistance even in the absence of cellulose production.

It remained possible that the ability to produce cellulose and the resulting biofilm architecture allowed AR3110 cells to better access exogenous DNA, perhaps by lysing neighboring cells to free DNA as has been shown in *S. pneumoniae* (17). If this were true, then it would follow that the increase in frequency of double antibiotic-resistant cells observed in AR3110 co-cultures might result from the presence of more donor DNA in the environment. To test this possibility, the macrocolonies from the four cell-gDNA mixes propagated on YESCA agar supplemented with Congo red and $CaCl_2$ were collected into LB liquid medium, serially diluted and then plated on double selective plates as described above. In the absence of any antibiotics, progeny from AR3110 amp cells mixed with W3110 cam genomic DNA and AR3110 cam cells mixed with W3110 amp genomic DNA were observed to grow well, resulting in 10^8 to 10^9 cfu/ml. Of these, ~10^2 cfu/ml and ~10^3 cfu/ml were also able to grow in the presence of both ampicillin and chloramphenicol for AR amp × gDNA W cam and AR cam × gDNA W amp mixes, respectively (Figure 5A). Five colonies from AR amp × W cam dilution and three colonies from AR cam × W amp were serially passaged on fresh double selective plates, P1. AR amp × gDNA W cam progeny streaked out to single colonies, while AR cam × W amp progeny was unable to grow (Figure 5B). To confirm the presence of double antibiotic resistance was stable, two colonies from P1 of the AR amp × W cam mix was serially passed on fresh double selective plates, P2, resulting in single colonies for both isolates (Figure 5C).Similar to what I found with AR amp × gDNA W cam mixes, mixing W3110 amp cells with genomic DNA from W3110 cam produced stable double antibiotic-resistant colonies that could be serially passaged, while mixing W3110 cam cells with genomic DNA from W3110 amp gave no growth on ampicillin and chloramphenicol plates similar to AR cam × W amp (data not shown). Table 2 shows the average frequency of double antibiotic-resistant cells obtained for each cell-genomic DNA combination across all passages and trials.

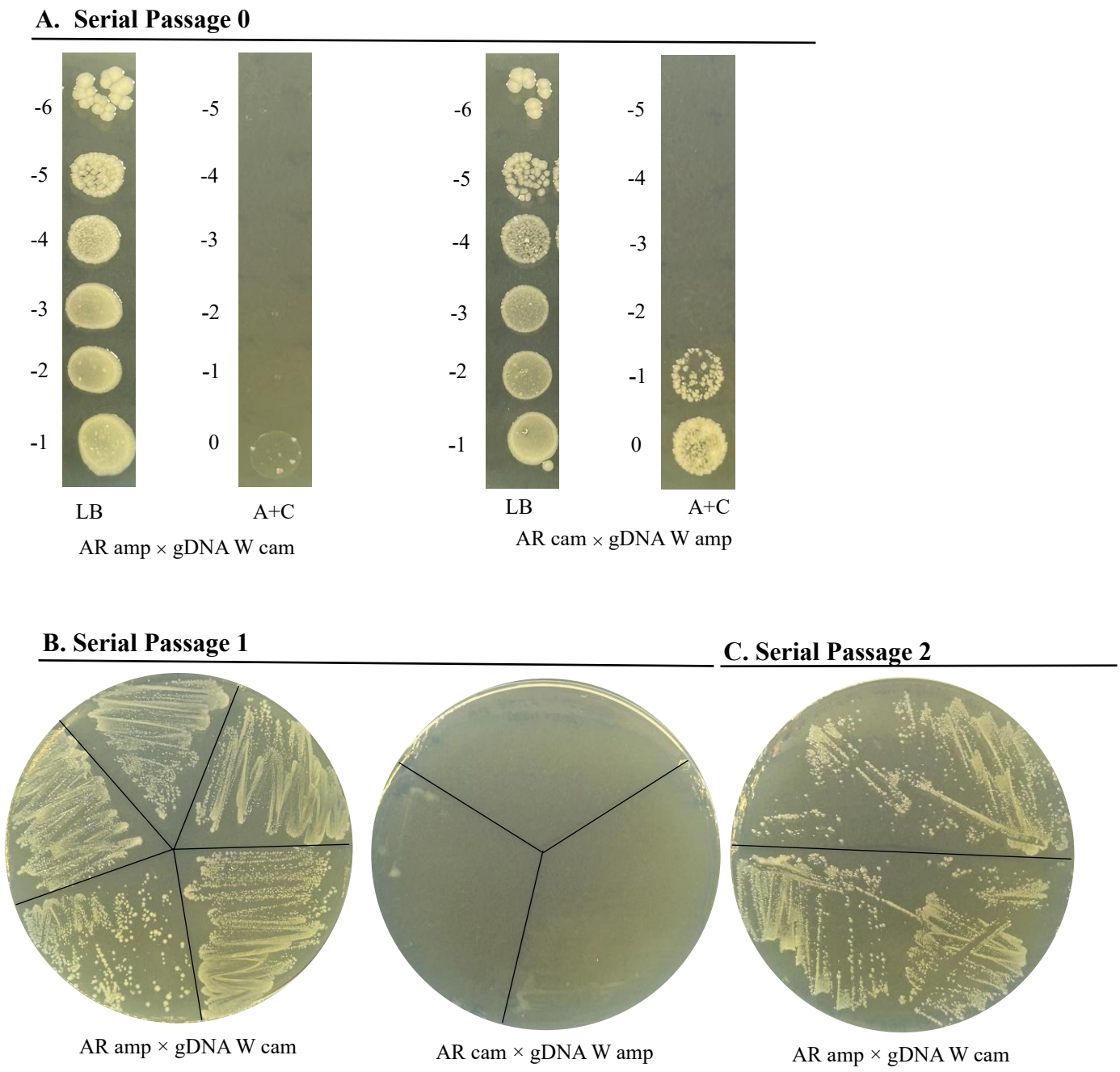


Figure 5. *E.* coli cells mixed with purified genomic DNA are able to acquire and maintain double antibiotic resistance even in the absence of cellulose production. (A) Colonies from cell × purified genomic DNA mixes biofilm grown on YESCA supplemented with Congo red and $CaCl_2$ were collected in 1-ml LB medium and 10-fold serially diluted before duplicate 10-µl spots were placed on LB and LB supplemented with ampicillin and chloramphenicol agar plates and incubated for 24 hours at 37˚C (serial passage 0, P0). (B) Single colonies that grew on double antibiotic selective plates from P0 were transferred to fresh ampicillin and chloramphenicol agar plates and streaked to isolation. Representative plates from serial passage 1 (P1) after being incubated for 24 hours at 37˚C are displayed. (C) Single colonies from P1 were serially passaged on fresh ampicillin- chloramphenicol agar plates and streaked to isolation (serial passage 2, P2).

Table 2. Frequency of ampicillin-chloramphenicol double resistant colonies from cell × purified genomic DNA mixes grown on YESCA with Congo red and $CaCl_2$ agar.

Cell × purified genomic DNA mixes	Average number of amp-cam resistant cells per million at passage 0*	First Serial Passage		Second Serial Passage	
		Number of colonies plated	Number fully grown out to isolated colonies**	Number of Colonies Plated	Number fully grown out to isolated colonies**
AR amp × gDNA W cam	32	8	6	5	5
W amp × gDNA W cam	26	7	5	3	2
AR cam × gDNA W amp	27	3	0	-	-
W cam × gDNA W amp	40	3	0	-	-

*Average of at least 2 independent experiments shown.

** The presence of isolated colonies was assessed after 24 hours of growth at 37˚C.

PCR with agarose gel analysis of ampicillin and chloramphenicol-resistant progeny from inter-strain co-cultures and cell-DNA mixes.

The serially passaging results suggested that genetic transfer might have occurred between AR3110 amp x AR3110 cam cells in co-culture and when AR3110 amp or W3110 amp cells were mixed with genomic DNA from W3110 cam to produce double antibiotic-resistant cells. To verify the presence of these antibiotic resistance markers in their genomes and show stable genetic transformation, I used PCR to amplify the ampicillin or chloramphenicol cassette. The ampicillin cassette is linked to the *E. coli recF* gene, while *xonA* is tagged with the chloramphenicol marker. In the case where *recF* contains the ampicillin resistance gene, I expected a PCR fragment of 6.3 kb, while the absence of ampicillin produces a 1.3 kb fragment. For *xonA* gene containing the chloramphenicol resistance cassette, I expected a 2.9 kb PCR fragment and in its absence a 1.8 kb fragment. Progeny from two independent, serially passaged AR3110 *recF*::amp × AR3110 *xonA*::cam co-cultures; one each of serially passaged cell-genomic DNA mixes of AR3110 *recF*::amp × gDNA W3110 *xonA*::cam and W3110 *recF*::amp × gDNA W3110 *xonA*::cam; and single mutant strains AR3110 *recF*::amp and AR3110 *xonA*::cam were analyzed by PCR. As shown in Figure 6A, all progeny from co-cultures and cell-DNA mixes contained the ampicillin gene at 6.3 kbp similar to what was observed for AR3110 *recF*::amp single mutant. None of the progeny from co-cultures and cell-DNA mixes appeared to contain the chloramphenicol resistance marker at 2.9 kbp seen in the single AR3110 *xonA*::cam mutant but instead gave multiple bands at wild type 1.8 kbp and non-specific amplification at 2.4 kbp (Figure 6B), suggesting either these cells have *xonA* wild-type gene or these cells carry an extrachromosomal piece of DNA. I also prepared genomic DNA from one of my serially passaged AR3110 amp x AR3110 cam co-cultures for whole genome sequencing. While the

sequencing data showed the presence of the ampicillin resistance cassette, I was unable to detect the chloramphenicol gene (data not shown).

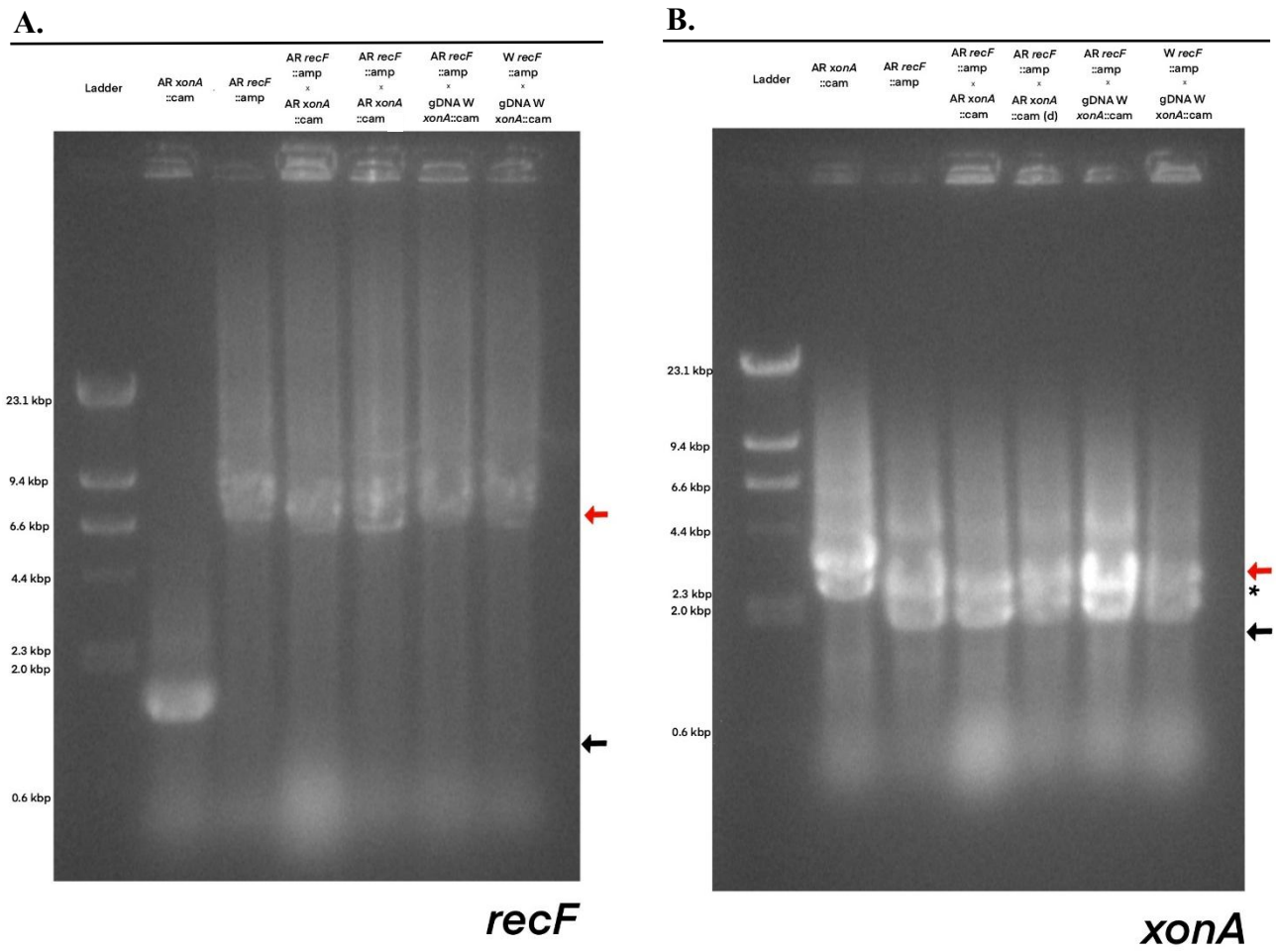


Figure 6. Agarose gel analysis of the *recF* and *xonA* gene of ampicillin and chloramphenicol-resistant progeny from inter-strain co-cultures and cell-DNA mixes. PCR reactions using AR *xonA*::cam, AR *recF*::amp, AR *recF*::amp × AR *xonA*::cam, AR *recF*::amp × AR *xonA*::cam, AR *recF*::amp × gDNA W *xonA*::cam, and W *recF::*amp × gDNA W *xonA*::cam as templates and (A) *recF* and (B) *xonA*- specific primers were separated on an agarose gel as shown. Red arrows indicate the predicted migration pattern for the antibiotic resistance marker tagged genes, black arrows represent the migration pattern for wild-type genes, and the asterisk represents non-specific amplification.

Discussion

This study explored the role of biofilm formation in the exhibition of natural competence in *E. coli*. While previous studies have identified growth conditions known to induce natural competence for *E. coli,* none have directly investigated whether biofilm formation could present an alternative pathway for horizontal gene transfer. My results suggest the cell's ability to synthesize cellulose in the presence of calcium supplementation both changes the architecture of

biofilms and contributes to the acquisition of exogenous DNA from the environment, at least transiently.

I observed that pdar morphology on the periphery of the biofilm was dependent on at least one partner in a co-culture being able to synthesize cellulose and the presence of calcium in the growth medium. In the absence of cellulose biosynthesis or calcium supplementation, biofilms were smooth and glossy. Consistent with these observations, the formation of ridges and wrinkles, which give the periphery of biofilms its characteristic pdar morphology, has been previously reported to require both curli fibers and cellulose in monocultures (13). Interestingly, pdar macrocolonies arise even when only one strain in a mixed culture synthesizes cellulose, suggesting that this molecule drives the organization of biofilms whether it is composed of a single strain or multiple strains. In contrast, cellulose biosynthesis was required in both partners in a co-culture to consistently produce stable genetic transfer, indicating the cellulose biosynthesis proficiency does influence the ability of exogenous DNA uptake which is supported by existing research (18).

The colonies from cell-genomic DNA mixes displayed both pdar at the periphery and tangled fibers at the center of the biofilm when a cellulose-producing strain was involved in the mix, but only when calcium was also present in the medium. Calcium ions are known to induce natural competence in *E. coli* (2), while curli amyloid protein has been shown to irreversibly bind DNA fibers during biofilm formation in *Salmonella* Typhimurium (6). This would suggest that cellulose production in combination with curli-DNA composites could have a direct influence on the transformation ability of the biofilms produced by cell-genomic DNA mixes. However, I found there was no difference in the ability of cellulose-proficient compared to cellulose-deficient strains in producing progeny with hybrid antibiotic resistance when mixed with exogenous DNA. Instead, the appearance of stable hybrids was dependent on whether the intact strain used carried the ampicillin or chloramphenicol resistance gene rather than its ability to produce cellulose. It is of note that the ampicillin resistance cassette disrupts the *recF* gene, while the chloramphenicol resistance marker tags a *xonA* (*sbcB*)-HA fusion. It has been previously shown that purified RecF protein by itself inhibits strand exchange between homologous DNA in vitro (4), so that a *recF* mutant might be predicted to affect recombination. On the other hand, the *Vibrio* SbcB homolog appears to facilitate natural transformation and recombination in this bacterial species (5). One potential modification for further research would be to relocate the antibiotic resistance markers used to genes or intergenic regions unrelated to recombination, allowing focus on the process of biofilm formation itself.

Based on these experiments, I found the transfer of antibiotic resistance is a low frequency event dependent on the contributing members which is also supported by previous studies (14). Unexpectedly, I was unable to detect the presence of both antibiotic resistance genes in hybrids exhibiting ampicillin- and chloramphenicol-resistance using either PCR or whole-genome sequencing despite being able to serially passage these cells on doubly selective plates. This would indicate a potential transient or non-recombinant transfer of genetic markers. Further

research is required to understand the mechanism by which these inter-strain co-cultures and cell-DNA mixes exhibit double antibiotic resistance.

Overall, my results suggest that the natural competence of *E. coli* through the acquisition of antibiotic resistance markers is influenced by cellulose proficient biofilms and the presence of calcium chloride supplementation.

References

1. Abebe, G. M. (2020). The Role of Bacterial Biofilm in Antibiotic Resistance and Food Contamination. International Journal of Microbiology, 2020, 1–10. https://doi.org/10.1155/2020/1705814
2. Baur, B., Hanselmann, K., Schlimme, W., & Jenni, B. (1996). Genetic transformation in freshwater: Escherichia coli is able to develop natural competence. Applied and Environmental Microbiology, 62(10), 3673–3678. https://doi.org/10.1128/aem.62.10.3673-3678.1996
3. Courcelle, J., Crowley, D. J., & Hanawalt, P. C. (1999). Recovery of DNA Replication in UV-Irradiated Escherichia coli Requires both Excision Repair and RecF Protein Function. Journal of Bacteriology, 181(3), 916–922. https://doi.org/10.1128/JB.181.3.916-922.1999
4. Courcelle, J., Ganesan, A. K., & Hanawalt, P. C. (2001). Therefore, what are recombination proteins there for? BioEssays, 23(5), 463–470. https://doi.org/10.1002/bies.1065
5. Dalia, T. N., & Dalia, A. B. (2024). SbcB facilitates natural transformation in Vibrio cholerae in an exonuclease-independent manner. Journal of Bacteriology, e00419-24. https://doi.org/10.1128/jb.00419-24
6. Gallo, P. M., Rapsinski, G. J., Wilson, R. P., Oppong, G. O., Sriram, U., Goulian, M., Buttaro, B., Caricchio, R., Gallucci, S., & Tükel, Ç. (2015). Amyloid-DNA Composites of Bacterial Biofilms Stimulate Autoimmunity. *Immunity*, *42*(6), 1171–1184. https://doi.org/10.1016/j.immuni.2015.06.002
7. Jeffries, J., Thongsomboon, W., Visser, J. A., Enriquez, K., Yager, D., & Cegelski, L. (2021). Variation in the ratio of curli and phosphoethanolamine cellulose associated with biofilm architecture and properties. Biopolymers, 112(1), e23395. https://doi.org/10.1002/bip.23395
8. Lorenz, M. G., & Wackernagel, W. (1994). Bacterial Gene Transfer by Natural Genetic Transformation in the Environment. MICROBIOL. REV., 58.
9. Michaelis, C., & Grohmann, E. (2023). Horizontal Gene Transfer of Antibiotic Resistance Genes in Biofilms. Antibiotics, 12(2), 328. https://doi.org/10.3390/antibiotics12020328
10. Olsén, A., Jonsson, A., & Normark, S. (1989). Fibronectin binding mediated by a novel class of surface organelles on Escherichia coli. *Nature*, *338*(6217), 652–655. https://doi.org/10.1038/338652a0
11. Palchevskiy, V., & Finkel, S. E. (2006). Escherichia coli Competence Gene Homologs Are Essential for Competitive Fitness and the Use of DNA as a Nutrient. Journal of Bacteriology, 188(11), 3902–3910. https://doi.org/10.1128/JB.01974-05
12. Palchevskiy, V., & Finkel, S. E. (2009). A Role for Single-Stranded Exonucleases in the Use of DNA as a Nutrient. Journal of Bacteriology, 191(11), 3712–3716. https://doi.org/10.1128/JB.01678-08
13. Serra, D. O., Richter, A. M., Klauck, G., Mika, F., & Hengge, R. (2013). Microanatomy at Cellular Resolution and Spatial Order of Physiological Differentiation in a Bacterial Biofilm. mBio, 4(2), e00103-13. https://doi.org/10.1128/mBio.00103-13

14. Serra, D. O., Richter, A. M., & Hengge, R. (2013). Cellulose as an Architectural Element in Spatially Structured Escherichia coli Biofilms. Journal of Bacteriology, 195(24), 5540–5554. https://doi.org/10.1128/JB.00946-13
15. Serra, D. O., & Hengge, R. (2014). Stress responses go three dimensional – the spatial order of physiological differentiation in bacterial macrocolony biofilms. Environmental Microbiology, 16(6), 1455–1471. https://doi.org/10.1111/1462-2920.12483
16. Sinha, S., & Redfield, R. J. (2012). Natural DNA Uptake by Escherichia coli. PLoS ONE, 7(4), e35620. https://doi.org/10.1371/journal.pone.0035620
17. Steinmoen, H., Knutsen, E., & Håvarstein, L. S. (2002). Induction of natural competence in Streptococcus pneumoniae triggers lysis and DNA release from a subfraction of the cell population. Proceedings of the National Academy of Sciences, 99(11), 7681–7686. https://doi.org/10.1073/pnas.112464599
18. Sun, D., Zhang, Y., Mei, Y., Jiang, H., Xie, Z., Liu, H., Chen, X., & Shen, P. (2006). Escherichia coli is naturally transformable in a novel transformation system. FEMS Microbiology Letters, 265(2), 249–255. https://doi.org/10.1111/j.1574-6968.2006.00503.x
19. Römling, U. (2005). Characterization of the rdar morphotype, a multicellular behaviour in Enterobacteriaceae. Cellular and Molecular Life Sciences, 62(11), 1234–1246. https://doi.org/10.1007/s00018-005-4557-x
20. Ziemba, C., Shabtai, Y., Piatkovsky, M., & Herzberg, M. (2016). Cellulose effects on morphology and elasticity of Vibrio fischeri biofilms. Npj Biofilms and Microbiomes, 2(1), 1. https://doi.org/10.1038/s41522-016-0001-2
21. Zogaj, X., Nimtz, M., Rohde, M., Bokranz, W., & Römling, U. (2001). The multicellular morphotypes of Salmonella typhimurium and Escherichia coli produce cellulose as the second component of the extracellular matrix. *Molecular microbiology*, *39*(6), 1452–1463. https://doi.org/10.1046/j.1365-2958.2001.02337.x