Investigating the antibiofilm activity and dsDNA interactions of the established antimicrobial peptide indolicidin and derivatives.

Benjamin Sinclair[1,2]

April 30, 2026

Thompson Rivers University

Chem 4480: Directed Studies in Chemistry

Supervisor: Dr. Heidi Huttunen-Hennelly[1]

Co-Supervisor: Dr. Eric Bottos[2]

[1]Department of Physical Sciences (Chemistry)

[2]Department of Biological Sciences

Pages: 19

Abstract

Antibiotic-resistant biofilms present a major challenge in healthcare due to their enhanced tolerance to antimicrobials and other stressors. Antimicrobial peptides (AMPs) have emerged as promising alternatives to conventional antibiotics because of their broad-spectrum activity and rapid action. Indolicidin is a previously reported, cationic and tryptophan rich AMP which has strong antimicrobial activity but also hemolytic activity. Previous studies have replaced its various Trp residues with alanine to reduce hydrophobicity and hemolysis. This study investigated the antibiofilm activity and DNA-binding capability of indolicidin and four alanine containing derivatives: Δ2,3, Δ2,5, Δ1,4, and Δ4,5. These were tested against biofilms of *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Candida albicans* in a minimum biofilm eradication concentration (MBEC) assay. Peptide/DNA interactions were investigated by an electrophoretic mobility shift assay (EMSA) with agarose gels.Indolicidin exhibited the strongest antibiofilm activity, achieving at least 50% reduction in media optical density (OD) at concentrations of 50–100 ppm and reducing viable biofilm CFUs by multiple orders of magnitude. However, treated biofilms remained viable, diminishing practicality. Among the derivatives, Δ2,3 demonstrated the greatest antibiofilm activity, achieving 50% OD reduction of all species at 400 ppm. Δ1,4, Δ 2,5, and Δ4,5 showed limited activity despite previously reported as effective against planktonic cultures. Overall, all derivatives were less effective against biofilms than indolicidin, suggesting that reduced hydrophobicity from alanine substitution compromises antibiofilm activity. There were minimal differences in AMP activity between the Gram-negative *E. coli,* Gram-positive MRSA, and the fungus *C. albicans*, though activity against *C. albicans* was consistently the highest. EMSA results demonstrated clear interaction between indolicidin and double-stranded DNA, increasing at higher peptide-to-DNA mass ratios. Δ4,5 showed weaker binding and other derivatives did not show binding but were not tested above a 1:1 mass ratio. The results suggest that tryptophan residues are critical for DNA binding. Additionally, the observed reduction in DNA band intensity at high peptide concentrations suggests possible competition between indolicidin and DNA intercalating stains.

Introduction

Biofilms are bacterial communities with one or many species that are adhered to a surface and are generally more resistant to antibiotics and other stressors than normal colonies. They have a protective extracellular matrix which acts as a barrier against mechanical and chemical threats. The matrix is made of secreted polysaccharides, proteins and nucleic acids collectively referred to as extracellular polymeric substances (EPS; Shree et al., 2023; Agrawal, 2023; Wood, 2009). Biofilm formation can be initiated simply by cell adherence to a surface, and cell-cell signalling, both of which stimulate EPS gene expression (Agrawal, 2023; Shree et al., 2023). The growth beneath the matrix is stratified, with higher activity cells toward the surface, and more dormant cells which tend to persist through stressors, underneath (Shree et al., 2023). The high antibiotic resistance of biofilms is due to multiple factors including fast, efficient, and widespread horizontal gene transfer (especially of antibiotic resistance genes), inefficient diffusion of antibiotic molecules through the matrix, and faster production of antibiotic inhibitors or efflux pumps (Agrawal, 2023; Shree et al., 2023). The stratified cell growth structure also produces a heterogeneous metabolic environment, where dormant, slow metabolizing cells of the inner layers are highly protected by the matrix and other cells (Shree et al., 2023). Because of their toughness, biofilms pose problems in healthcare settings where they can grow on instruments or in the body. However, not all biofilms are pathogenic and some are engineered for use in bioremediation, biopower generation or chemical synthesis (Shree et al., 2023).

The existence of antibiotic resistant pathogens (including pathogenic biofilms) is a growing problem for public health and has worsened as traditional antibiotics are constantly overprescribed. As an alternative to traditional antibiotics, many antimicrobial peptides (AMPs) derived from natural or artificial sources have been researched. AMPs are characterized by their broad-spectrum activity, fast action, and difficult development of resistance, but also their tendency for cytotoxic and hemolytic effects (Matheson et al., 2013; Mercer-Brunelle, 2024; Podorieszach & Huttunen-Hennelly, 2010). The generally proposed mode of action for AMPs is membrane disruption and/or penetration into the cell, where they may bind nucleic acids or proteins to disrupt cell processes (Azad et al., 2011; Huang et al., 2010). The amount and order of hydrophobic and cationic amino acids are the main factors that govern both antimicrobial activity and hemolysis of a potential AMP. When designing or optimizing an AMP it is these

amino acids, specifically tryptophan, arginine, and lysine, which are the most relevant (Matheson et al., 2013; Podorieszach and Huttunen-Hennelly, 2010; Azad et al, 2011; Huang et al, 2010).

Mechanism of interaction with and penetration of the lipid bilayer by AMPs relies on the amount of tryptophan and other hydrophobic residues but also the dynamic structures of the short peptides. They may take multiple conformations beyond a standard α-helix, or oligomerize with other peptide molecules, to better partition into the hydrophobic membrane core (Huang et al., 2010; Podorieszach & Huttunen-Hennelly, 2010). Detergent-like activity can permeabilize the membrane, or multi-molecular complexes can form unselective pores in the membrane (Figure 1) through which more peptide can enter the cytoplasm and disrupt cell processes (Huang et al., 2010; Yeaman & Yount, 2003). The number of cationic residues of an AMP, specifically arginine and lysine, contribute to efficient action against bacteria since the negatively charged cell walls (phosphates of Gram positives; lipoteichoic acids of Gram negatives) promote diffusion of AMPs to the plasma membrane.

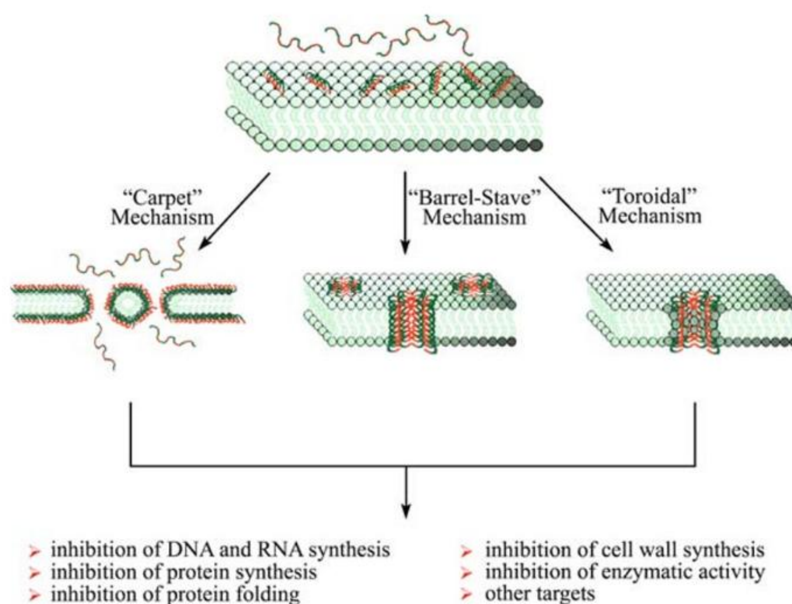

Figure 1. Mechanisms of pore complex formation by AMPs (Huang et al., 2010).

The effectiveness of AMPs against biofilm is less researched, and the testing process of new AMPs usually prioritises minimum inhibitory concentration (MIC) tests and hemolysis assays (Podorieszach and Huttunen-Hennelly, 2010; Mercer-Brunelle, 2024). However, it is

established that many AMPs also act as biofilm inhibitory peptides (BIPs) which can target a biofilm matrix and/or biofilm genes specifically (Agrawal, 2023).

This project will focus on testing the antibiofilm capabilities of the established AMP indolicidin and some of its derivatives. Indolicidin is a 13 amino acid AMP found naturally in bovine neutrophils and which has been studied extensively. It is amidated at the C-terminus and characterized by a +4 charge at physiological pH, along with a high tryptophan content (Hsu, 2005; Milne, 2013).

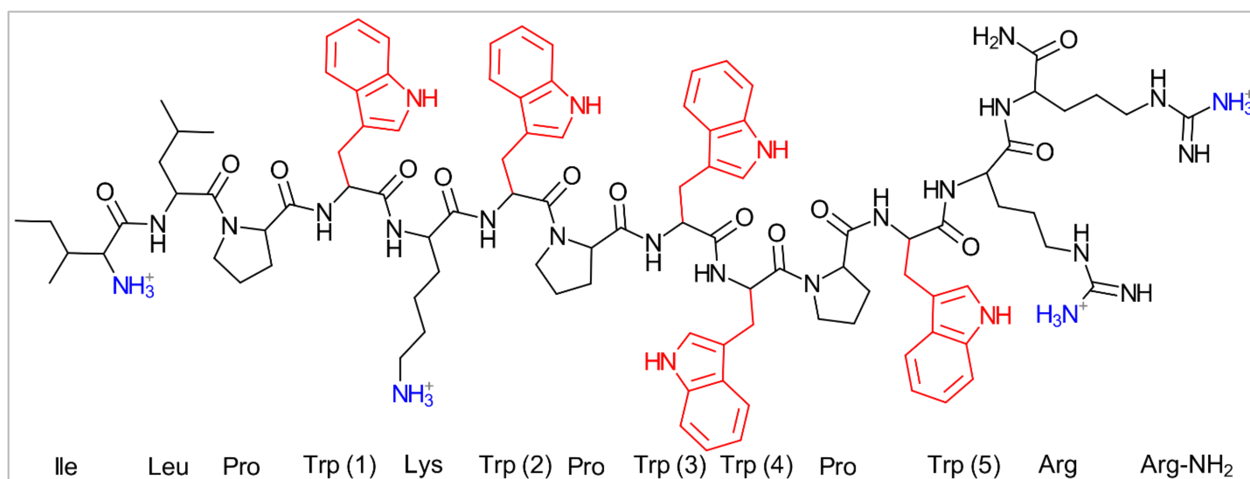

Figure 2. Structure of the AMP indolicidin.

While indolicidin has strong antimicrobial activity, it also displays significant hemolysis, leading to the development and testing of derivatives (Mercer-Brunelle, 2024; Podorieszach & Huttunen-Hennelly, 2010). Replacement of one or multiple of the five tryptophan residues with alanine was hypothesized to reduce the hydrophobicity enough to eliminate hemolysis while maintaining antimicrobial activity (see Table 1). Replacement of three or more tryptophan residues reduces apparently reduces hydrophobicity too much for membrane penetration, eliminating antimicrobial activity (Podorieszach & Huttunen-Hennelly, 2010). Derivatives which replaced tryptophans 2 and 3 (Δ2,3) or 4 and 5 (Δ4,5) maintained the highest level of antimicrobial activity with minimal hemolysis (Mercer-Brunelle, 2024). The derivatives listed in Table 1 are tested here for antibiofilm activity against *Escherichia coli,* Methicillin resistant *Staphylococcus aureus* (MRSA), and *Candida albicans*.

Table 1. Sequences and antimicrobial activity of Indolicidin and four derivatives. Antimicrobial activity was evaluated in previous research by minimum inhibitory concentration (MIC) assays on planktonic cultures. Data: Mercer-Brunelle, 2024; Podorieszach & Huttunen-Hennelly, 2010

Peptide	Sequence	Antimicrobial Activity	Hemolytic Activity
Indolicidin	ILPWKWPWWPWRR-NH_2	High	High
Δ2,3	ILPWKAPAWPWRR-NH_2	Moderate	Minimal
Δ2,5	ILPWKAPWWPARR-NH_2	Moderate	Minimal
Δ1,4	ILPAKWPWAPWRR-NH_2	Moderate	Minimal
Δ4,5	ILPWKWPWAPARR-NH_2	High	Minimal

As mentioned above, a common mode of action for AMPs is to bind proteins or nucleic acids once in the cytoplasm. Studies have established the dsDNA binding capability of indolicidin using an electrophoretic mobility shift assay (EMSA; Hsu, 2005; Mercer-Brunelle, 2024). Given the importance of secondary structure in indolicidin activity (Hsu, 2005; Huang et al., 2010), identification of residues or motifs critical to a certain function is important for future peptide design. An example is indolicidin's two Trp-Pro-Trp (WPW) motifs which are known to be critical in protein binding and secondary structure formation (Hsu, 2005; Podorieszach & Huttunen-Hennelly, 2010). The DNA binding capability of some indolicidin derivatives has been evaluated, with correlation back to antimicrobial activity (Mercer-Brunelle, 2024) however, interpretation of the EMSA used can be challenging and there is opportunity for optimization or redesign of the assay. In this experiment, the DNA binding capability of indolicidin and four derivatives is assessed by EMSA with observations compared to previous experiments (Hsu, 2005; Mercer-Brunelle, 2024).

Methods

Preparation of AMP solutions

Commercially synthesized peptides were obtained as solids of purity ~95% or greater and dissolved in sterile deionized water to a known concentration. Peptide concentration was determined using a NanoDrop One Spectrophotometer.

Minimum Biofilm Eradication Concentration Assay

Three microorganisms, methicillin resistant *Staphylococcus aureus, Candida albicans,* and *Escherichia coli* were re-streaked from subcultures on Mueller-Hinton (MH) agar plates to obtain isolated colonies. Isolated colonies were suspended in sterile phosphate-buffered saline (PBS) to the optical density of the McFarland standard ($OD_{621\ nm} = 0.11 \pm 0.02$). This yielded suspensions containing approximately 1.5×10^8 CFU/mL, which were then diluted to ~ 1×10^5 CFU/mL in MH broth and used for inoculation of the biofilm growth plate (*Innovotech* MBEC). Plates were inoculated with 150 µL of the appropriate suspension in each well, and incubated on a shaker for 24 – 32 hours at 37 °C and 110 rpm. To reduce evaporation of media in the biofilm growth wells, beakers of water were included to maintain humidity in the incubator. After incubation, biofilm growth was assessed by breaking off two growth check (GC) pegs for each organism. Staining of the pegs with crystal violet followed by light microscopy was used to visually verify biofilm formation. An inoculum for serial dilution and spot plating quantification of the biofilm growth was prepared by rinsing the second broken peg in PBS for 10 seconds, and then sonicating a microcentrifuge tube containing 200 µL of MH broth and the peg at 40 kHz for 30 minutes to dislodge the biofilm cells into suspension. The biofilm challenge plate was prepared with two-fold serial dilutions of the tested AMP in MH broth, with three replicates of each concentration per microorganism. There were also at least three growth control wells and one positive control well per microorganism, alongside three sterile wells per plate. The positive control well contained 740 ppm chloramphenicol in MH broth. The optical density at 621 nm of each well in the challenge plate was recorded, and then the biofilm peg lid was incubated in the challenge plate for 16 hours under the previous conditions. After incubation of the challenge plate, the peg lid was immediately transferred to a plate containing recovery media, which consisted of MH broth supplemented with surfactants (2% saponin, 1% Tween-80 w/v%) and 2.5 v/v% of a universal neutralizer (5% L-histidine, 5% L-cysteine, 10% reduced glutathione w/v%).

The challenge plate (without peg lid) and recovery plate (with peg lid) were allowed to equilibrate to room temperature. The optical density at 621 nm of each well in the challenge plate was then recorded for determination of growth inhibition by comparison to the initial optical density. The recovery plate was then sonicated at 30 kHz for 30 mins to dislodge the biofilm cells into suspension. The viable cell concentration of each well of the recovery plate can then be determined by serial dilution and spot plating. The cell concentration of peptide treated wells can be compared to growth control wells for determination of antibiofilm activity. The recovery plate can also be incubated to assess the viability of recovered biofilm cells.

Electrophoretic mobility shift assay

To assess DNA binding capability of short AMPs, varying concentrations of peptide was incubated with dsDNA and then visualized by gel electrophoresis. The DNA used was the plasmid pBluescript (pBS). In the first experiment, 200 ng of circular pBS was incubated with 10 ng, 50 ng, 100 ng, or 200 ng of Δ4,5 and Δ1,4 in a 10 μL volume of nuclease free water. After incubation for ~5 min at room temperature, each sample along with a no-peptide control was mixed with 2 μL of 6x loading dye and loaded into a 1% agarose gel in Tris-Acetate EDTA (TAE; 40 mM Tris-Acetate, 1mM EDTA) with 1x RedSafe nucleic acid stain. The gel was run at 120 V for ~60 mins. For the subsequent runs, the pBS was first linearized using EcoR1 in 20 μL reactions with 2 μL 10x FastDigest buffer, 1 μg pBS, and nuclease free water. The digest was completed by 1 hour incubation at 37 °C. In the second experiment, 200 ng of linear pBS was incubated with 50 ng or 200 ng of indolicidin and Δ2,5 in a 10 μL volume of nuclease free water. These along with a no-peptide control were incubated and loaded into a 1% gel as above which was run at 80 V for ~80 minutes. For the third experiment, 200 ng, 400 ng, or 1000 ng of indolicidin and Δ4,5 were incubated with 200 ng of linearized pBS in 10 μL volume. These were loaded into a 0.7% agarose gel and run for 90 V for ~60 minutes. The ladder used in all gels was 1kb Plus DNA Ladder RTU. In the third experiment, recovery of the DNA and peptide was attempted from various band locations by 'freeze-and-squeeze' gel extraction. Gel bands were excised and then placed in a filter pipet tip in a 1.5 mL tube, frozen at -80 °C for 10 minutes, then centrifuged at max speed for 5 minutes. The supernatant was analyzed for DNA and peptide concentration using the Nanodrop spectrophotometer.

Result

Minimum Biofilm Eradication Concentration Assay

The biofilm inhibition activity of five AMPs was assessed against biofilms of three microorganisms. Biofilms were grown by inoculating broth media in a 96-well plate, with a pegged lid providing the growth surface. To verify biofilm growth before peptide treatment, diagnostic pegs were removed and visualized by staining and light microscopy. Images representative of average or characteristic growth across all experiments are shown in Figure 3. Biofilm growth was consistent across all experiments and relatively consistent between microorganisms. Overall, there was only moderate growth, and coverage of the pegs was heterogeneous with some empty regions (Figure 3). The MRSA biofilms had the least growth in general, showing more heterogeneity (Figure 3B). The *C. albicans* pegs often had good coverage with a thin cell layer observed, as well as the regions with more growth (Figure 3C).

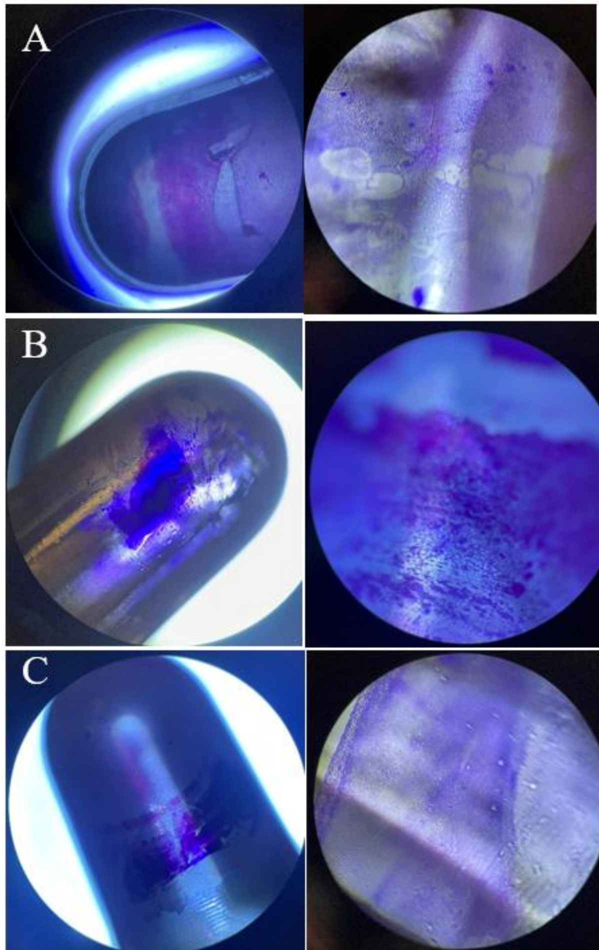

Figure 3. Characteristic biofilm growth images of A) *E. coli,* B) MRSA, and C) *C. albicans*.

The biofilm peg lid was then incubated in a peptide-containing challenge plate, with the turbidity of each well used to quantify the extent of biofilm propagation into the broth. A challenge plate containing 25 – 400 ppm of the peptide Δ2,5 is shown in Figure 4, with a minor decrease in turbidity from the growth control to 400 ppm wells of all three species. The positive control well for each species contained 740 ppm chloramphenicol, and demonstrates the turbidity of a sterile well.

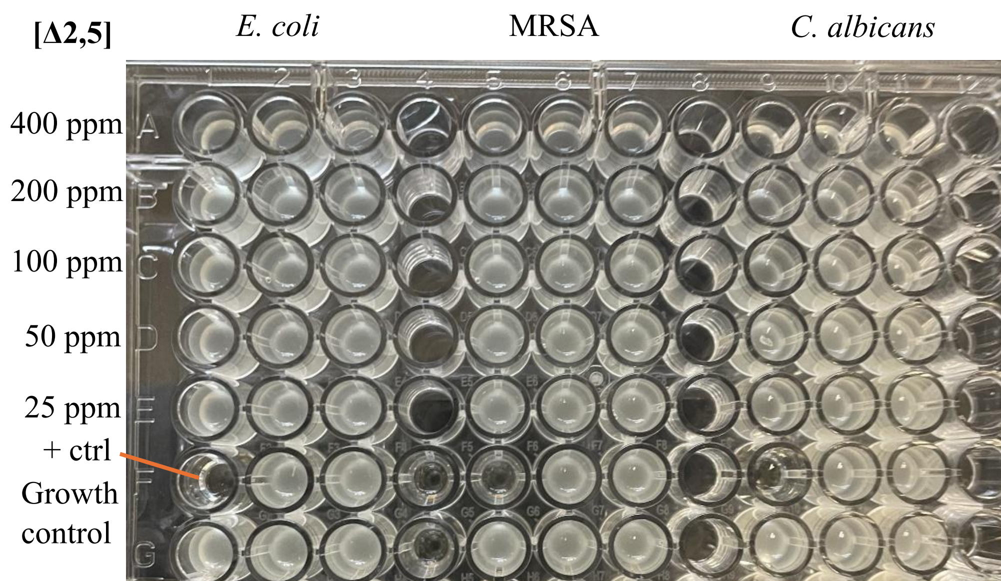

Figure 4. Challenge plate wells containing MH broth cultures after incubation with the biofilm peg lid and varying concentrations of peptide Δ2,5. Growth control wells had no peptide, and positive control wells had 740 ppm chloramphenicol.

The optical density of each well was measured to quantitatively describe the growth and peptide concentrations which caused at least 50% OD reduction are reported in Table 2. Indolicidin performed the best with concentrations of 50 ppm against MRSA and *C. albicans* and 100 ppm against *E. coli*. Of the derivatives, only Δ2,3 reached 50% OD reduction at the highest concentration tested (400 ppm) against all three organisms. Except for Δ2,5 against *C. albicans*, no other peptides achieved 50% OD reduction. The inhibition of each peptide at 400 ppm is described in Table 3. Indolicidin attained >80% OD reduction for all three microorganisms, the only peptide with promising antibiofilm activity. Δ2,3 performed the best of the derivatives, with ~60% OD reduction for each species while Δ1,4 performed the worst. Interestingly, the three

least active derivates, Δ2,5, Δ1,4, and Δ4,5, all had the most activity against *C. albicans*, and all derivates were the least active against the Gram-negative *E. coli*. Though inhibition was observed, the four indolicidin derivatives do not have significant biofilm eradication activity. Furthermore, all AMPs performed worse than previously reported MICs against planktonic cultures (Mercer-Brunelle, 2024; Podorieszach & Huttunen-Hennelly, 2010).

Table 2. Minimum concentration which resulted in 50% decreased OD_{621} of three microorganisms biofilm growth media compared to control. A dash indicates the peptide did not show a 50% OD_{621} decrease at the highest concentration tested (400 ppm).

Peptide	*Escherichia coli*	Methicillin resistant *Staphylococcus aureus*	*Candida albicans*
Indolicidin	100 ppm	50 ppm	50 ppm
Δ2,3	400 ppm	400 ppm	400 ppm
Δ2,5	-	-	400 ppm
Δ1,4	-	-	-
Δ4,5	-	-	-

Table 3. OD_{621} of biofilm growth media compared to control of the highest concentration peptide treatment (400 ppm). A value of 100% would indicate no decrease in growth and a value of 0% would indicate a sterile well.

Peptide	*Escherichia coli*	Methicillin resistant *Staphylococcus aureus*	*Candida albicans*
Indolicidin	9.4%	17%	4.4%
Δ2,3	41%	36%	39%
Δ2,5	60%	54%	48%
Δ1,4	87%	83%	58%
Δ4,5	76%	67%	54%

While the data above reports the inhibition of growth in the broth, it does not measure the activity against cells in the biofilm itself. Given the activity of indolicidin reported in Tables 2 & 3, the biofilm inhibition was then determined by colony forming units (CFUs). These figures represent only cells in the biofilm structure on the peg surface. Though only a minimum value was determined for the growth controls, results show indolicidin treatment reduced CFUs by multiple orders of magnitude against all three organisms. Despite this, the treated biofilms remained highly viable, having >10^4 CFUs and exhibiting growth in the recovery plate.

Table 4. Activity of Indolicidin against biofilms of three microorganisms reported as post-treatment CFUs. Biofilm cells were recovered from the pegs into recovery media by sonication, followed by estimation of CFUs by serial dilution and spot plating. A greater than sign indicates colonies were too concentrated to quantify at the dilutions used.

	Growth Control estimated CFUs	**200 ppm Indolicidin estimated CFUs**	**100 ppm Indolicidin estimated CFUs**
E. coli	>4 x 10^7	10^5	10^6
MRSA	>4 x 10^7	No Data	5 x 10^4
C. albicans	>4 x 10^7	10^5	>10^6

Electrophoretic mobility shift assay

To assess the DNA binding capability of indolicidin and the four derivatives, electrophoretic mobility shift assays (EMSA) were conducted using agarose gels. Three runs were completed with slightly varied conditions each time. The first run (Figure 5A) had samples of 10 ng – 200 ng Δ4,5 or Δ1,4 incubated with 200 ng of circular pBluescript (pBS) plasmid DNA. pBS is ~3kb in size, however it appears at ~3.9 kb due to its circular structure. No shift in electrophoretic mobility was observed between the no peptide (NP) control and the AMP treatments (Figure 5A). Linearized pBS generated by restriction digestion was used for the second run (Figure 5B) causing the NP control to appear at 3 kb. There was some mobility shift observed in the 200 ng indolicidin sample, with a narrow band at 3 kb compared to control and some DNA visible remaining in the well. This suggests that indolicidin is interacting with the dsDNA to prevent its migration either structurally or by neutralizing the charge. Indolicidin and

Δ4,5 were then tested again in higher amounts (200 – 1000 ng) using linearized pBS (see Figure 5C). A circular pBS sample was included to verify the restriction enzyme linearization, and both the supercoiled (~2.2 kb) and non-supercoiled (~3.5 kb) bands were observed. The linear NP control band at 3 kb confirms the successful linearization. The same narrower band was observed in the 200 ng indolicidin treatment as in the previous run, but there was less DNA visible in the well. The 400 ng band was even smaller, and the 1000 ng band was barely visible (Figure 5C). For Δ4,5, the 200 ng and 400 ng bands do not differ from the NP control, however the 1000 ng band is smaller and shows faint smearing. At 1000 ng, both peptides are visibly interacting with the dsDNA but indolicidin clearly has a greater effect. The disappearance of the 1000 ng indolicidin band is interesting and suggests the peptide is interfering with the RedSafe DNA stain. A gel extraction was performed from a variety of bands on the third gel, but no DNA or protein was detected in the supernatant.

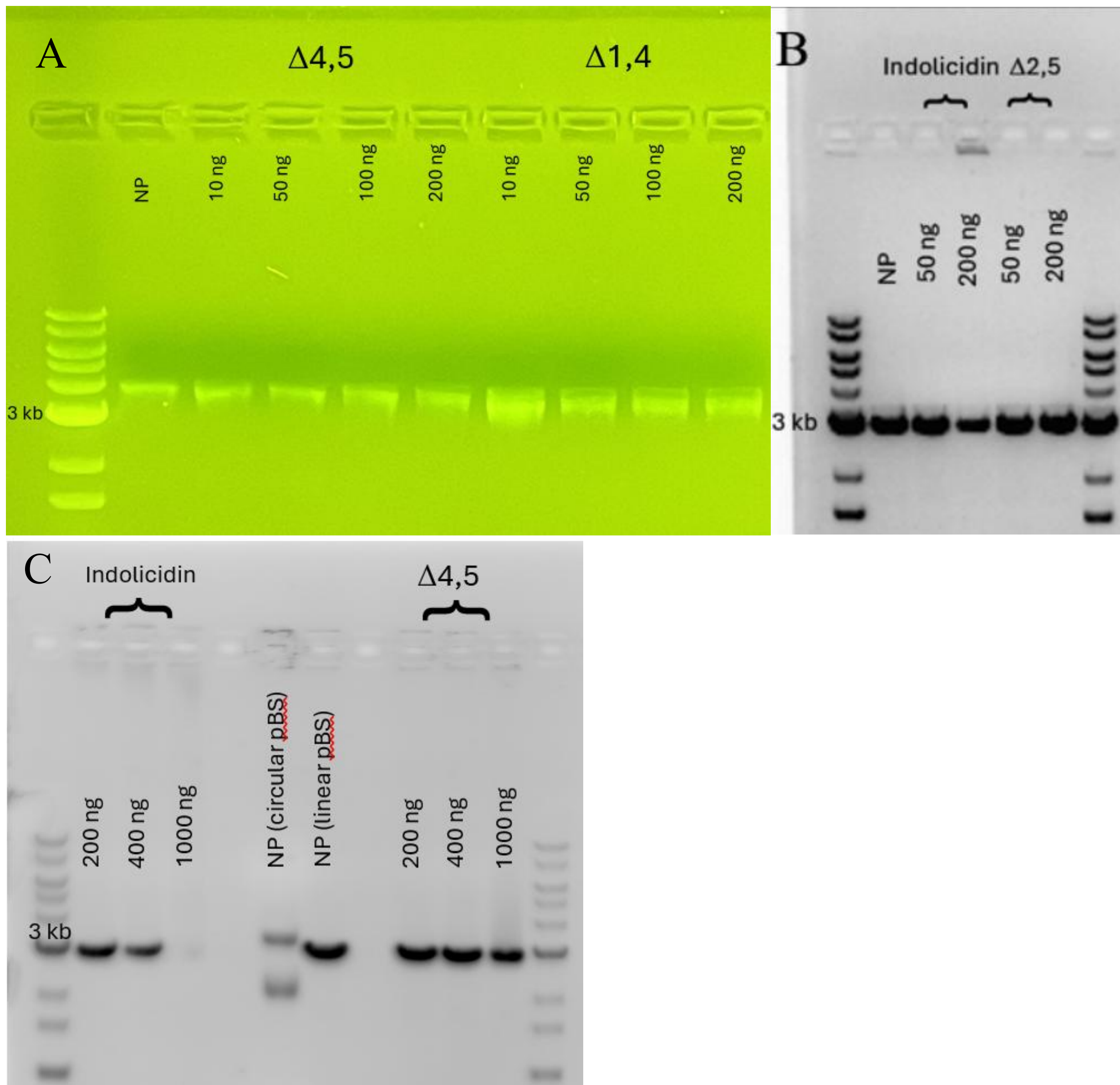

Figure 5. Agarose gel electrophoresis of 200 ng pBS incubated with varying amounts of indolicidin and its derivatives.

Discussion

Though conclusive results were obtained from the MBEC assay, there is opportunity for improving the methodology to increase data quality. Obtaining good biofilm growth on the peg lid before exposure to peptides contributes to accuracy and precision. Despite small changes to the growth conditions (i.e. incubation time), complete homogenous biofilm coverage of the pegs was not achieved in this study. With different microorganisms forming biofilms differently (Shree et al., 2023), it is possible that growing three different species on the same plate under the same conditions is impractical. A major concern with this assay is contamination, especially during diagnostic steps where pegs need to be broken off and the lid is exposed. Maintaining strict aseptic technique, and inclusion of sterile media control wells is important.

The main method of growth quantification used here was $OD_{621\ nm}$ measurements of the 96-well challenge plate. Biofilm cells propagate into the broth and form planktonic colonies, the inhibition of which is measured by turbidity. This is similar to a traditional MIC, where a planktonic broth culture is the starting point (Mercer-Brunelle, 2024; Podorieszach & Huttunen-Hennelly, 2010). This method is quick and effective but does not allow determination of activity against the biofilm structure itself. Despite that, comparison between these assays and MICs against planktonic cultures gives insight into the resistance allowed by biofilms.

Indolicidin achieved 50% OD reduction at 50 ppm against MRSA and *C. albicans* and 100 ppm against *E. coli* (see Table 2), but was previously reported to achieve a 90% OD reduction at 50 ppm against planktonic colonies of these organisms (Mercer-Brunelle, 2024). Similarly, 400 ppm Δ2,3 had about 60% OD reduction here (Table 3), but achieved 90% OD reduction at 250 ppm against planktonic MRSA and *C. albicans* (Mercer-Brunelle, 2024). Biofilms appear to give the most resistance against Δ4,5, which achieved only ≤46% OD reduction here, but previously performed better than indolicidin against planktonic colonies (Podorieszach & Huttunen-Hennelly, 2010). One consideration is that different organisms' biofilms could be more or less tolerant to AMPs (Shree et al., 2023), which could reconcile the observed higher activity against *C. albicans* (see Table 3). It is hypothesized that a biofilm's extracellular matrix provides structural protection from AMPs by slowing diffusion to the cell membrane or by EPS binding and sequestering a peptide molecule. Given the general structure and mode of action of AMPs, it is unlikely the fast horizontal gene transfer or increased inhibitor

production of biofilms contributes to AMP resistance as it would to traditional antimicrobials (Huang et al., 2010; Shree et al., 2023). Given that only indolicidin had promising antibiofilm activity, the reduced hydrophobicity of alanine-containing derivatives appears detrimental to antibiofilm activity. However, alanine substitutions are necessary to maintain minimal hemolysis.

To quantify activity against the biofilm itself, individual pegs or the peg lid can be sonicated to recover cells into a suspension. Serial dilution and spot/spread plating allows CFU/peg determination. This can be used to quantitatively compare biofilm growth between assays and species, which increases reproducibility and gives better growth validation. Comparison of biofilm CFUs between a peptide-treated and negative control peg gives a better quantification of a peptide's activity than OD. Indolicidin was found to decrease CFUs by multiple orders of magnitude at 100 ppm against MRSA, and 200 ppm against *E. coli* and *C. albicans*. Despite that, the treated biofilms still had $>10^4$ CFUs and remained viable, exhibiting growth in the recovery plate. Though AMPs have been reported to target biofilm structures/genes specifically (Agrawal, 2023), these results do not support that activity for indolicidin. Given the lower activity of the indolicidin derivatives, their reduction of biofilm CFUs was not reported.

Given the relevance of DNA binding for AMP mode of action, an EMSA was conducted with indolicidin and derivatives. This assay has been previously performed with indolicidin, however there are opportunities for its improvement. Plasmid DNA was used as the template, which is known to take multiple structures when circularized. Supercoiled plasmid runs faster than expected on a gel, while the relaxed or 'nicked' circular form runs slower (see Figure 5C; Corless & Gilbert, 2017). To ensure any AMP binding is not specific to a supercoiled plasmid structure, and so that the no-peptide control shows only one band, the plasmid is linearized using a restriction enzyme before peptide incubation.

Indolicidin showed clear interaction at a peptide/DNA mass ratio of 1:1, with more interaction at a 5:1 ratio. Δ4,5 also shows interaction at a 5:1 ratio, but not at 2:1, suggesting indolicidin has a higher DNA binding affinity (see Figure 5). Δ1,4 and Δ2,5 were tested at a maximum 1:1 mass ratio but showed no evidence of DNA interaction. The affinity of indolicidin compared to Δ4,5 suggests tryptophan residues contribute to binding. The results here disagree with a previous assay where indolicidin showed interaction at a 0.2:1 mass ratio (Hsu, 2005), but indolicidin and the derivative results agree with Mercer-Brunelle's (2024) results of the same

assay. More experiments with higher mass ratios of all derivatives are needed to determine specific residues contributions to DNA binding.

The principle of this assay is that AMP binding increases the molecular weight of the dsDNA, and the band will run observably slower on the gel than a no-peptide control. This result has been directly observed in the EMSA with indolicidin derivatives reported by Mercer-Brunelle (2024) however the Hsu (2005) report yielded different observations, with the peptide/DNA complex remaining in the well and the band intensity decreasing with increasing peptide concentration. It is hypothesized that signal intensity decreases with increasing peptide concentration because the DNA stain used in gels (commonly ethidium bromide, SYBR Safe, or RedSafe), and tryptophan-containing AMPs both bind dsDNA by intercalating between the stacked nitrogenous bases. This is good evidence for AMP intercalation and suggests high importance of tryptophan residues in DNA binding. Future experiments could explicitly test this hypothesis by using another method of DNA visualization like 3' addition of fluorescent nucleotides by a terminal deoxynucleotide transferase (TdT) enzyme.

Another interpretation challenge is the band visible still in the well, suggesting the peptide prevented DNA movement completely. Initially it was hypothesized that peptide molecules structurally block the agarose pores and preventing DNA migration, but migration of DNA at a 1:1 mass ratio (see Figure 5A) suggests this is not the case. Since indolicidin and its derivatives are cationic, the net charge of a peptide/DNA complex could be neutral, positive, or negative. A negative complex would give the expected result described above and seen by Mercer-Brunelle (2024). A neutral complex could reconcile the loss of DNA electrophoretic mobility seen here and by Hsu (2005) and would suggest cationic residues are neutralizing the negative phosphates of DNA in a 1:1 ratio. It follows that one peptide molecule spans 4 base pairs of one strand of the dsDNA, suggesting more immobile DNA would be observed at higher peptide/DNA mass ratios. A high pH electrophoresis buffer could be used in future experiments to reduce the positive charge of the AMPs and examine the effect on DNA binding.

Literature Cited

Agrawal, R. (2023). *Biofilms*. https://research.ebsco.com/c/amz5ui/ebook-viewer/pdf/xzgaw7gion/page/pp_49

Azad, M. A., Huttunen-Hennelly, H. E. K., & Ross Friedman, C. (2011). Bioactivity and the first transmission electron microscopy immunogold studies of short de novo-designed antimicrobial peptides. *Antimicrobial Agents and Chemotherapy*, *55*(5), 2137–2145. https://doi.org/10.1128/AAC.01148-10

Corless, S., & Gilbert, N. (2017). Investigating DNA supercoiling in eukaryotic genomes. *Briefings in Functional Genomics*, *16*(6), 379–389. https://doi.org/10.1093/bfgp/elx007

Hsu, C.-H. (2005). Structural and DNA-binding studies on the bovine antimicrobial peptide, indolicidin: Evidence for multiple conformations involved in binding to membranes and DNA. *Nucleic Acids Research*, *33*(13), 4053–4064. https://doi.org/10.1093/nar/gki725

Huang, Y., Huang, J., & Chen, Y. (2010). Alpha-helical cationic antimicrobial peptides: Relationships of structure and function. *Protein & Cell*, *1*(2), 143–152. https://doi.org/10.1007/s13238-010-0004-3

Matheson, S., Cheeptham, N., & Huttunen-Hennelly, H. (2013). Investigating the Effects of Hydrophobicity and Charge on the Therapeutic Ability of the Antimicrobial Histatin 8 Peptide for Potential Use in Oral Applications. *International Journal of Biology*, *5*(2), p85. https://doi.org/10.5539/ijb.v5n2p85

Mercer-Brunelle, A. (2024). *DETERMINING ANTIMICROBIAL SUSCEPTIBILITY AND HEMOLYTIC ACTIVITY OF INDOLICIDIN DERIVATIVES*. Thompson Rivers University.

Milne, T. (2013). *CHARACTERIZATION OF THE INTERACTIONS BETWEEN INDOLICIDIN AND ITS ANALOGS WITH LIPOPOLYSACCHARIDE THROUGH CAPILLARY ELECTROPHORESIS* [Master's]. Simon Fraser University.

Podorieszach, A. P., & Huttunen-Hennelly, H. E. K. (2010). The effects of tryptophan and hydrophobicity on the structure and bioactivity of novel indolicidin derivatives with promising pharmaceutical potential. *Organic & Biomolecular Chemistry*, *8*(7), 1679–1687. https://doi.org/10.1039/B921248E

Shree, P., Singh, C. K., Sodhi, K. K., Surya, J. N., & Singh, D. K. (2023). Biofilms: Understanding the structure and contribution towards bacterial resistance in antibiotics. *Medicine in Microecology*, *16*, 100084. https://doi.org/10.1016/j.medmic.2023.100084

Yeaman, M. R., & Yount, N. Y. (2003). Mechanisms of Antimicrobial Peptide Action and Resistance. *Pharmacological Reviews*, *55*(1), 27–55. https://doi.org/10.1124/pr.55.1.2