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Cytotoxic activity of colicin E1, E3 and E9 against *E.coli* BW25113 in the planktonic and biofilm states

Cleopatra O. Harry* and Daniel Walker

Faculty of Biomedical and Life Sciences, University of Glasgow, Scotland, United Kingdom

***Corresponding author e-mail:** *becleo2002@yahoo.com*

KEYWORDS

Anti-bacterial protein; cytotoxic activity; biofilms; planktonic cells.

A B S T R A C T

Colicins are plasmid-encoded 3-domain antibacterial proteins that are produced during times of stress by and active against *Escherichia coli* and closely related bacteria. The aim of this study was to develop protocols to test the cytotoxic activity of pore-forming colicins E1, RNases colE3 and DNases colE9 against non-pathogenic *E.coli* BW25113and to compare the efficiency of their MIC to kill cells that grow on agar plate, liquid cultures (as free-swimming cells) and solid surfaces (as biofilms embedded cells). If successful this protocol could be used as headway for *Pseudomonas eruginosa* which are pathogenic and produces pyocins which are bactericidal against strains of *Pseudomonas eruginosa*. In vitro experiment was carried out on the cytotoxic activity of the purified colicins against colicin- sensitive planktonic *E.coli* BW25113 using a cell killing assay at 37ºC. Biofilm killing assay was also developed and used against *E.coli*- biofilm cells. The result obtained showed that all three colicins tested were active against the strain BW25113 grown on agar plates and liquid culture as planktonic cells. However biofilm resistance to the cytotoxic activity of the enzymatic colicin ensued when tested against biofilms with little exception to the pore-forming colicins.

Introduction

Colicins are plasmid encoded antibacterial proteins which bind to a receptor on the surface of *E.coli* (Alice, 2002; Andreas *et al*., 2006; Boon, 1972). Colicin production is induced in a nutrient- limited environment [. Boon, T. [1971]4] or by high temperatures (Bouveret *et al*., Bowman *et al*., 1971). Colicins are produced by *Escherichia coli* and closely related bacteria during periods of stress to kill and inhibit the growth of those bacteria that are closely related to the producing strains (Brunden *et al*., 1984; Cascales *et al*., 2007). Colicins are not active against

more distantly related strains because these strains lack the receptor and/or translocation system for uptake of the colicins (Brunden *et al*., 1984). Colicins have been found to be present in approximately 30% of natural *E.coli* isolates.

Colicins are classified according to the cell surface receptor they bind: Colicins that bind the vitamin B12 receptor, BtuB, are known as the E group (Cavard, 1995). E-colicins are ~60KDa proteins in size and contain three functional domains which are related to the stages involved in the mechanism of cell killing (Ceri *et al*., 1999): (i) a central receptor- binding [R] domain that facilitates entry into the cell by binding to the outer membrane receptor of the target cell (Cascales *et al*., 2007, Cavard, 1995; Cerca, *et al*., 2005; Emma *et al*., 2007), (ii) an N-terminal translocation [T] domain that utilizes the Tol-dependent translocation system to mediate the translocation of the toxin across the membrane into the cell (Cavard, 1995). , Elkins *et al*., 2007, Garinot-Schneider *et al*., 1997, Google , 2009), (iii) and a C-terminal cytotoxic [C] domain that induces cell death by hydrolysis of nucleic acid or by pore formation in the cytoplamic membrane (Cascales *et al*., 2007). The colicins possess an immunity protein which confers resistance to its host. This immunity protein binds to the cytotoxic domain to inhibit and neutralise its activity, thereby protecting the producing cell from the lethal effect of both endogenous and exogenous colicins (Kimberly, 2004; Kuhar and Zgur-Bertok,1999). The immunity protein is specific to each colicin type (Kimberly, 2004; Kuhar and Zgur-Bertok,1999).

Biofilms are dense aggregates of surface-adherent micro-organisms in a community enclosed in an exopolysaccharide matrix (Lazdunski *et al*.,1998). According to Innovotech Incorporation, biofilms exist in virtually every natural environment and forms in response to the presence of solid surfaces and shear force (flow) as a mechanism to avoid being swept away from their surroundings. Centres for Disease Control and Prevention estimate that 65% of human bacterial infections involve biofilms. It has been demonstrated that the antimicrobial agents required to kill bacteria in its biofilm embedded state is 10-1000 times more concentration than the amount necessary to kill the same amount of free-swimming bacteria cells (Naoki *et al*., 2005; Ogawa *et al*., 1999; Ohno and Imahori, 1978).

The aim of this project was to develop protocol to test the cytotoxic activity of colicins E1, E3 and E9 against *E.coli* that grow in the biofilm state and to compare the efficiency in which these colicins kill cells that grow on solid media, liquid culture and solid surfaces (biofilms). Also to determine the Minimal Inhibitory Concentration (MIC) and Minimal Biofilm Eradication Concentration (MBEC) of colicin, that is the lowest concentration that will either inhibit growth (in planktonic state) or eradicate growth (in biofilm state) of the tested bacterium. In vitro experiment was carried out on the cytotoxic activity of purified colicin E1, E3 and E9 against colicin- sensitive planktonic *E.coli* cells using a cell killing assay at different concentrations in broth cultures and spot test assay on agar plates. Biofilm killing assay was also developed and used against *E.coli* biofilm embedded cells.

Materials and Methods

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surface of *E.coli* (Alice, 2002; Andreas *et al*., 2006; Boon, 1972). Colicin production is induced in a nutrient- limited environment [. Boon, T. [1971]4] or by high temperatures (Bouveret *et al*., Bowman *et al*., 1971). Colicins are produced by *Escherichia coli* and closely related bacteria during periods of stress to kill and inhibit the growth of those bacteria that are closely related to the producing strains (Brunden *et al*., 1984; Cascales *et al*., 2007). Colicins are not active against more distantly related strains because these strains lack the receptor and/or translocation system for uptake of the colicins (Brunden *et al*., 1984). Colicins have been found to be present in approximately 30% of natural *E.coli* isolates.

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purified colicin E1, E3 and E9 against colicin- sensitive planktonic *E.coli* cells using a cell killing assay at different concentrations in broth cultures and spot test assay on agar plates. Biofilm killing assay was also developed and used against *E.coli* biofilm embedded cells.

Result and Discussion

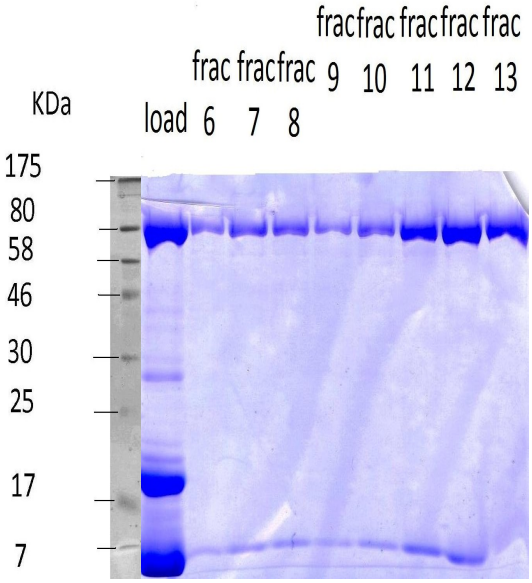
Plancktonic and biofilm cell killing assay were conducted to determine the minimal inhibitory and minimal biofilm eradication concentrations of the tested colicin in other to establish the lowest concentration that will inhibit cell growth and also stop inhibition. The cytotoxic activity of the colicin E1, E3 and E9 domains were tested against a wild typenon-pathogenic *E.coli* (BW25113) strain that was grown on LB agar plates, LB broth media as free swimming cells and LB broth media as surface attached or embedded cells.

Purification of Col E9

Colicin E9 and its immunity protein Im9 was purified using the Ni affinity column and gel filtration column as explained above. After gel filtration, the fractions containing the peaks assumed to contain the purified protein were collected and ran on a SDS page electrophoresis to determine which fraction contain the desired sized protein which is greater than 58kDa.

Lethality of Col E1, E3 and E9 on BW25113 cells grown on LB agar medium

Figure.1 SDS PAGE of the purified colicin E9. Fractions 11, 12 and 13 contain the desired sized protein (col E9). Fractions 6, 7, 8, 9 and 10 could be dimmers such as DNA, DNA polymerase, transcription factors etc. because of their thin bands as compared to the last three thick dense bands.



Agar plates were prepared by inoculation of the strain BW25113 into 0.7% non nutrient agar at 42ºC which was overlaid onto LB-agar plates and spotted with a 5 fold serial dilution of the colicin to be tested. The plates were incubated overnight at 37ºC. This strain was tested for sensitivity to the purified pore-forming colicin E1, ribonuclease type colicin E3 and endonuclease type colicin E9 in spot test assay. The cytotoxic domain activities of these colicins were assessed by visible inhibitory zones.

Fig.2 shows that the *E.coli* strain was sensitive to all 3 colicins but differs in the level of sensitivity when compared with colicin E1. Studies on the cytotoxic activities of these colicins have been reported by several researchers and this include Ying *et al*., (2008); Daniel *et al*., (2007, 2004) to kill related *Escherichia coli* strains on agar plates. From the result, the minimal inhibitory concentration of colicin E1, E3 and E9 was $1.2x10^{-2}$nM, $9.4x10^{-2}$nM and $7.5x10^{-2}$nM respectively. This

means that col E1 is approximately 10 fold or 10 times more active than col E3 and E9.

Lethality of Col E1, E3 and E9 on BW25113 cells grown in LB broth media

Under sterile condition, *E.coli* BW25113 was picked from agar plate and sub-cultured in LB broth media at an optimum temperature of 37ºC in a shaking incubator at a speed of 200rpm. Colicin was added to the medium after 2h of incubation and the absorbance was measured (OD_{600}) at an interval of 30min for the length of 4h 30min. Figures. 3, 4 and 5 clearly illustrates that cell growth was induced, reduced and inhibited in the presence of different concentrations of nuclease or pore-forming colicins as indicated in the figures below. Result obtained in Fig.3 shows that the MIC of the cytotoxic domain activity of the pore-forming colicin E1 was much more active when compared to the cytotoxic activity of ribonucleaseColicin E3 (Figure.4) and endonuclease colicin E9 (Figure.5). Concentrations below the MIC results in reduced cell number and growth above the control. The information deduced from this experiment was that col E1 is more powerful and efficient in cell killings than both colE3 and colE9 while col E3 is more efficient in cell killing than col E9. The efficiency of cell killing by DNasecolicin (col E9) has been reported by Walker *et al*., (2007) to be dependent on the level of anionic phospholipid in the target cell and the positive charge on the nuclease.

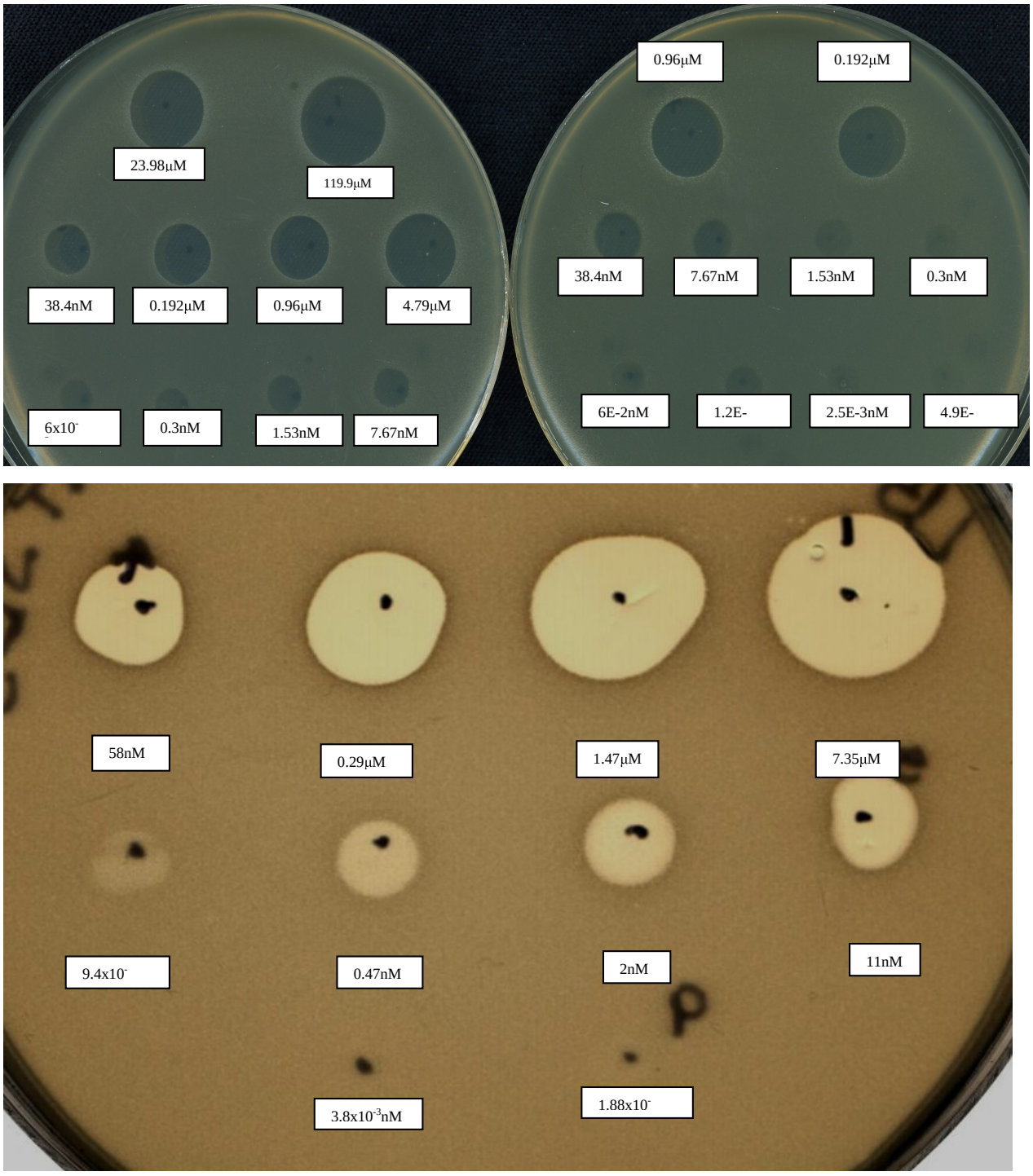
Biofilm cell killing assay on a solid surface

Biofilm killing was performed using the MBEC™ High-throughput (HTP) assay to determine the minimal biofilm eradication concentration of the colicin to be tested. Under sterile conditions, 1 in 30 dilution of a 1.0 McFarland standard was transferred into the MBEC™ device and incubated in both ambient (20-25°C) and optimum temperature (37°C) for 48h and 72h in other to allow time for bio-film maturation or establishment. The biofilms were exposed for 4h to the lethal action of a 1 in 5 fold dilution of the antimicrobial proteins to be tested. Crystal violet staining was done to stain cells that remained fixed after the toxic action of the colicin. These cells were extracted from the pegs with 70% ethanol and measured at a wavelength of 600nm using a spectrophotometer. Figures 6, 7, 8, 9 and 10 shows the response of biofilms to different dilutions of antimicrobial proteins used. The result obtained in Figures .6, 7 and 8 clearly illustrates that col E1, at a dilution of 1 in 5, completely inhibited the growth of biofilms grown in ambient temperature (between 20ºC and 25ºC) for 48h but technically failed to inhibit or had little effect on biofilms that were cultured for 3 days at ambient temperature and biofilms that were cultured for 2days at optimum temperature (37ºC). Figures .9 and 10 shows that biofilms are resistant to the action of RNase and DNasecolicins since these colicins had little or no effect on biofilm formation.

Col E1 showed activity against biofilms grown for 2 days at concentration of 23.98μM. The age of the biofilm can be critical in determining antibiotic resistance; therefore I tested biofilm grown for 3 days.

This experiment shows that the age of the biofilm is also an important factor in resistance to biofilm killing by colicin. BW25113 was then grown at optimum temperature in order to determine if temperature is critical in biofilm resistance to colicin action.

Figure.2 Cytotoxic spot plat assay showing *E. coli* BW25113 is sensitive to both pore-forming and enzyme colicins. A 5-fold serial dilution of colicin E1, E3 and E9 was made and spotted onto a growing lawn of the bacterium cells which were incubated overnight at 37ºC under static condition. Cytotoxicity was measured by the zones of inhibition observed. Clear zones indicate cell killing by the colicin and hazy zones indicates partial toxicity.



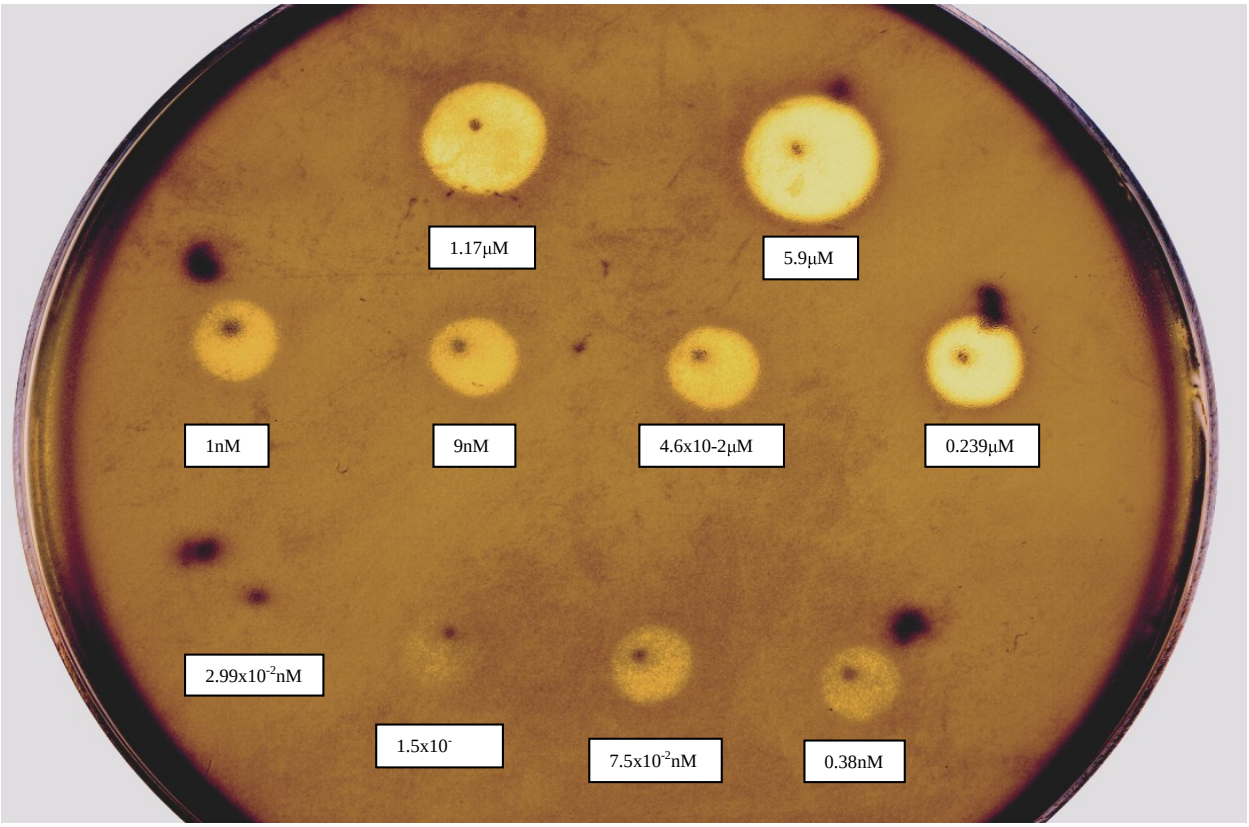


Figure.3 Cytotoxic activity of a pore-forming colicin E1 on E.coli BW25113 strain in liquid culture. Cell growth is inhibited at concentration of $1.98x10^{-2}$nM which is the colicin MIC for this bacterium. Concentrations below the MIC results in reduced cell growth and growth above the control. BW25113 was Sub-cultured for 2h to rapidly grow at 37ºC. Colicin E1 was added to the growing culture after 2h of incubation. Absorbance $OD_{600nm}$was measured at 30min intervals. This experiment was done twice.

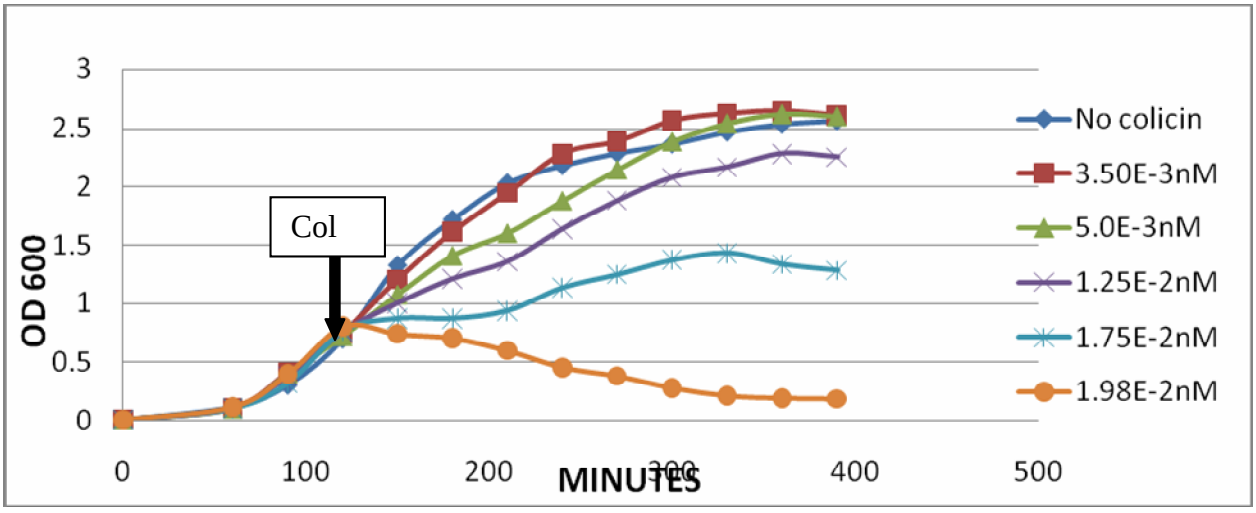


Figure.4 Cytotoxic activity of an RNasecolicin E3 on E.coli BW25113 strain in liquid culture. Cell growth is inhibited at concentration of 0.20nM which is the colicin MIC for this bacterium. Concentrations below the MIC results in reduced cell growth and growth above the control. BW25113 was Sub-cultured for 2h to rapidly grow at 37ºC. Colicin E3 was added to the growing culture after 2h of incubation. Absorbance $OD_{600nm}$was measured at 30min intervals. This experiment was done twice.

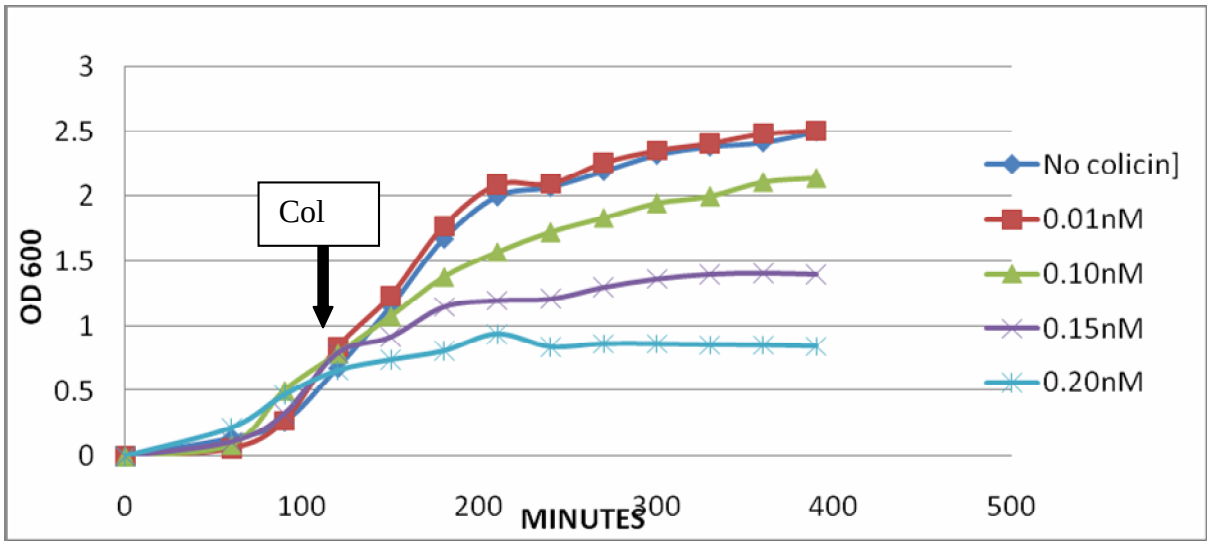


Figure.5 Cytotoxic domain activity of a DNasecolicin E9 on E.coli BW25113 strain in liquid culture. Cell growth is inhibited at concentration of 0.60nM which is the colicin MIC for this bacterium. Concentrations below the MIC results in reduced cell growth and growth above the control. BW25113 was Sub-cultured for 2h to rapidly grow at 37ºC. Colicin E9 was added to the growing culture after 2h of incubation. Absorbance $OD_{600nm}$was measured at 30min intervals. This experiment was done twice.

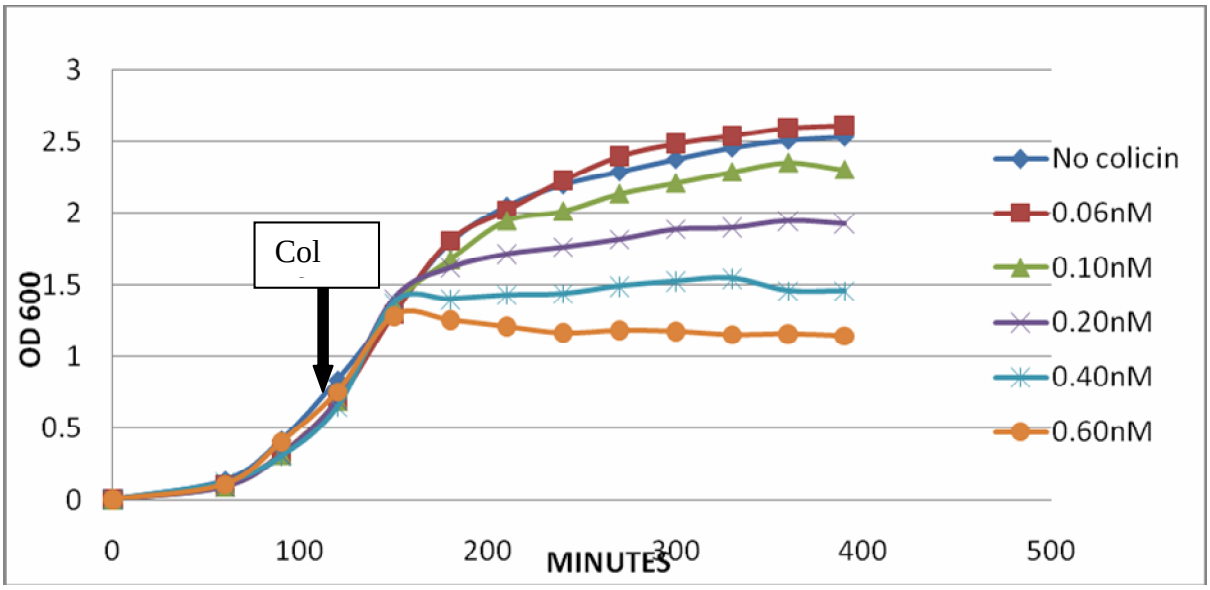


Figure.6 The lethal action of col E1 on biofilms, cultured at ambient temperature for 48h. Col E1 eradicated biofilm growth at a 23.98μM concentration in a 1 in 5 dilution. This serves as the MBEC for col E1. The effect of col E1 on biofilm formation ceased at 1 in 500 dilution, however biofilm growth was stimulated at 1 in 5000 dilution and thereafter impeded. 2 days cultured biofilms-attached pegs were exposed (for 4h at room temperature) to a 5 fold dilution of col E1. After which crystal violet staining was done to stain cells that remained fixed after the toxic action of the colicin. These cells were extracted from the pegs with 70% ethanol and measured at a wavelength of 600nm using a spectrophotometer.

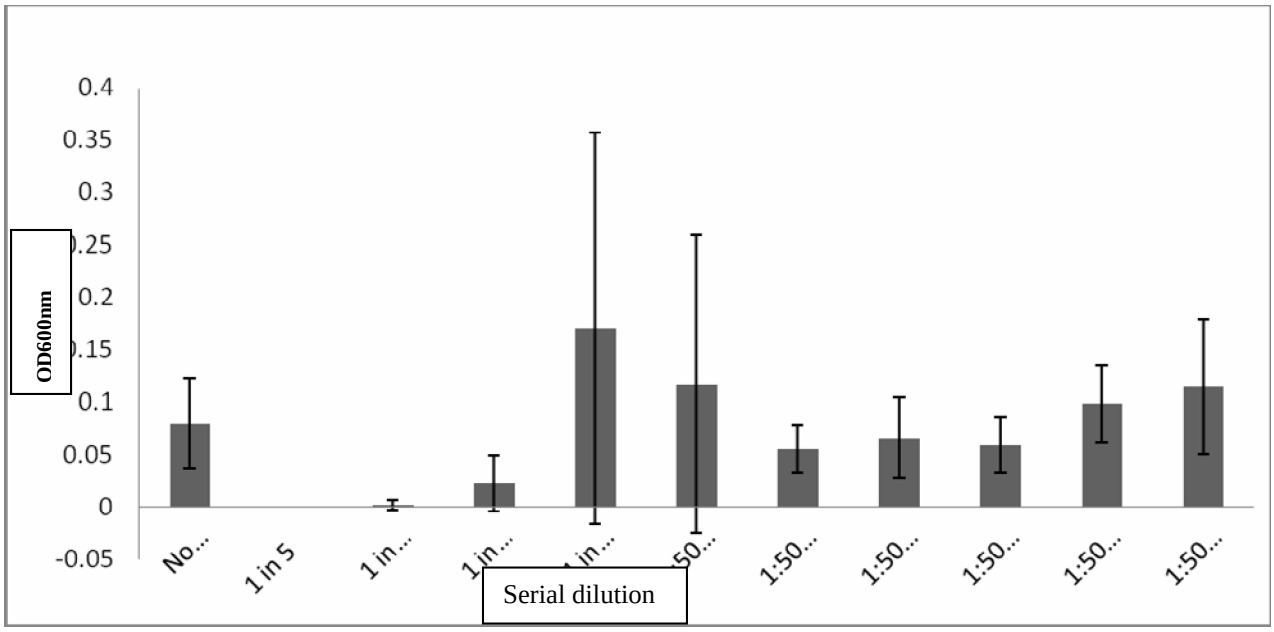


Figure.7 The lethal action of col E1 on biofilms, cultured at ambient temperature for 72h shows that the colicin has little or no effect, rather than inhibiting, stimulated biofilm growth. 3 days cultured biofilms-attached pegs were exposed (for 4h at room temperature) to a 5 fold dilution of col E1. After which crystal violet staining was done to stain cells that remained fixed after the toxic action of the colicin. These cells were extracted from the pegs with 70% ethanol and measured at a wavelength of 600nm using a spectrophotometer

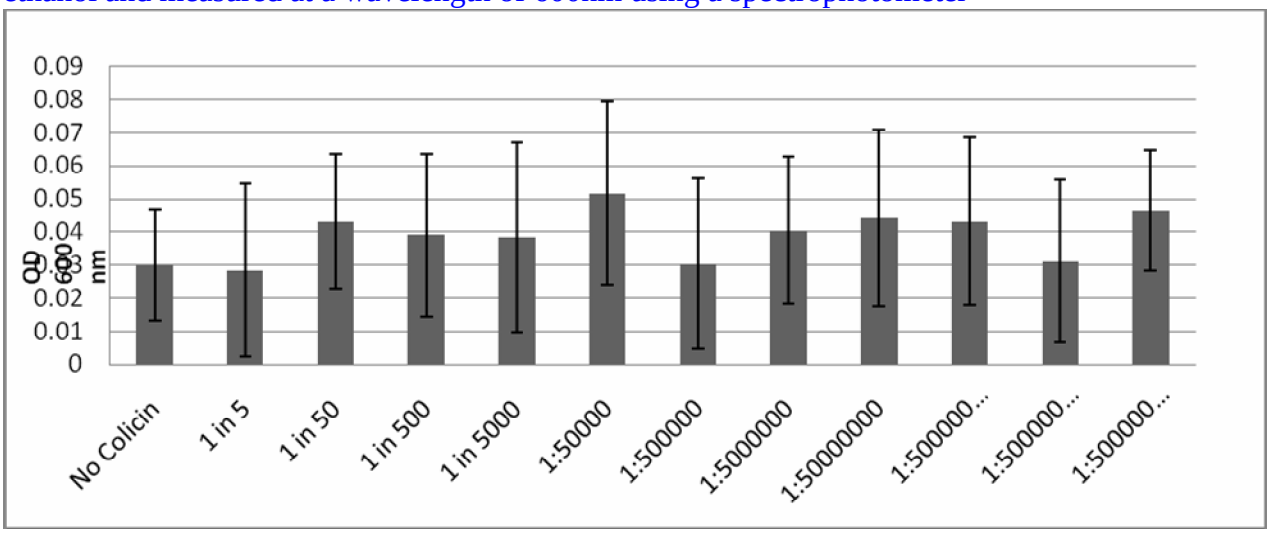


Figure.8 The lethal action of col E1 on biofilms, cultured at optimum temperature (37ºC) for 48h. Growth was reduced but not eradicated at concentration of 23.9µM contained in a 1 in 5 dilution. Biofilm growth was stimulated at 1 in 500 dilution and thereafter impeded. 2 days cultured biofilms-attached pegs were exposed (for 4h at room temperature) to a 5 fold dilution of col E1. After which crystal violet staining was done to stain cells that remained fixed after the toxic action of the colicin. These cells were extracted from the pegs with 70% ethanol and measured at a wavelength of 600nm using a spectrophotometer.

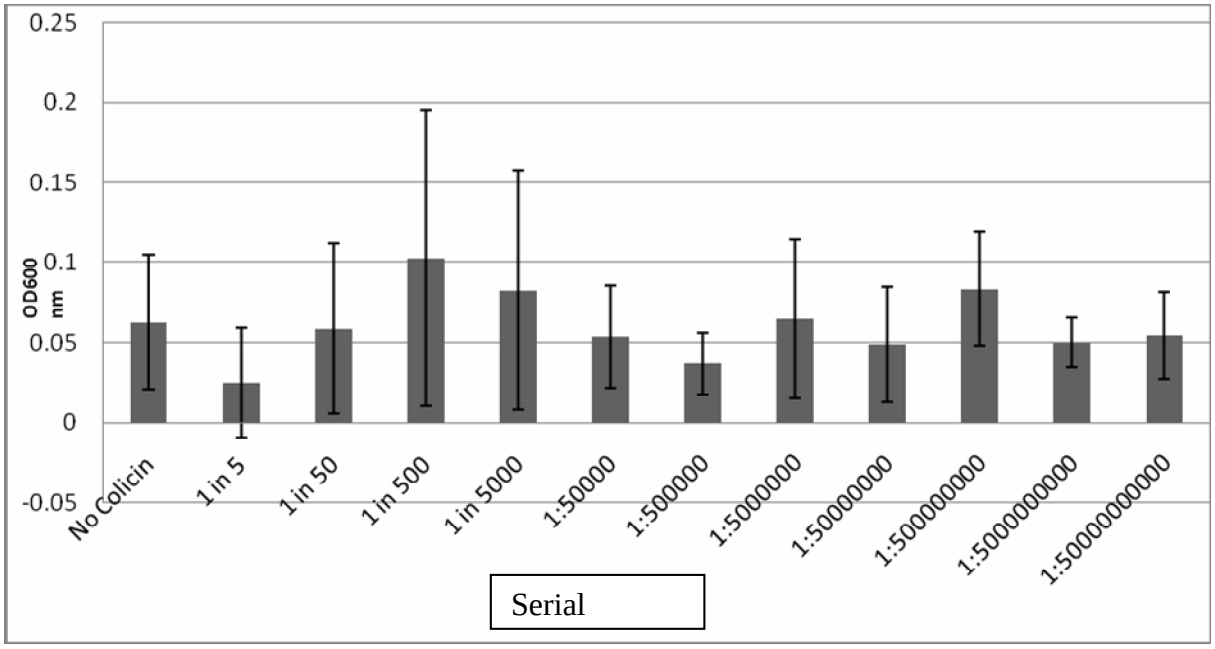


Figure.9 The lethal action of col E3 on biofilms, cultured at ambient temperature for 48h shows that the colicin has little or no effect, rather than inhibiting, stimulated biofilm growth. 2 days cultured biofilms-attached pegs were exposed (for 4h at room temperature) to a 5 fold dilution of col E3. After which crystal violet staining was done to stain cells that remained fixed after the toxic action of the colicin. These cells were extracted from the pegs with 70% ethanol and measured at a wavelength of 600nm using a spectrophotometer.

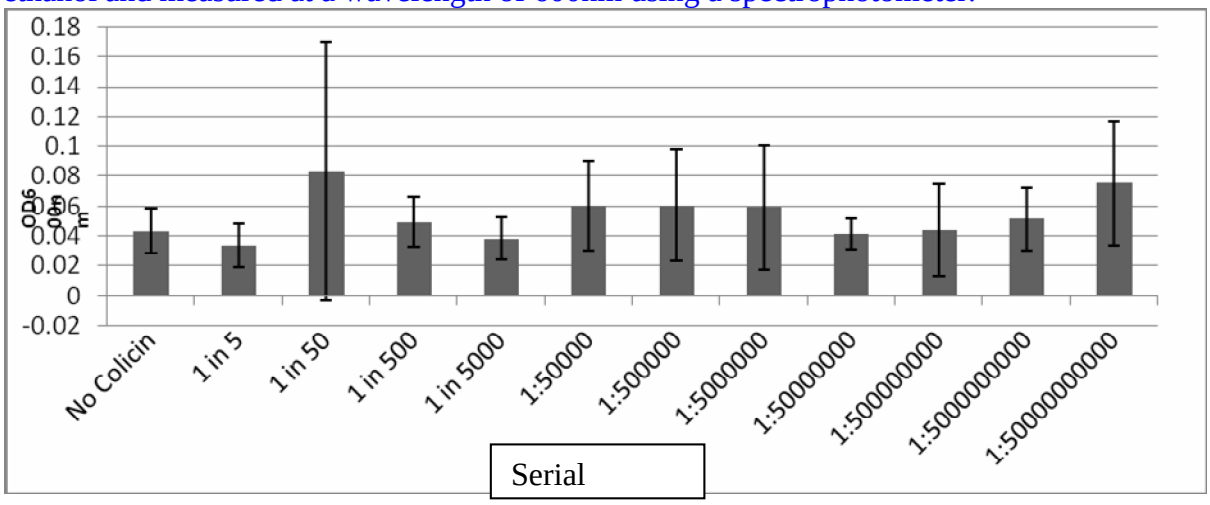


Figure.10 The lethal action of col E9 on biofilms, cultured at ambient temperature for 48h shows no significant reduction in growth, rather than inhibiting, stimulated biofilm growth. 2 days cultured biofilms-attached pegs were exposed (for 4h at room temperature) to a 5 fold dilution of col E9. After which crystal violet staining was done to stain cells that remained fixed after the toxic action of the colicin. These cells were extracted from the pegs with 70% ethanol and measured at a wavelength of 600nm using a spectrophotometer.

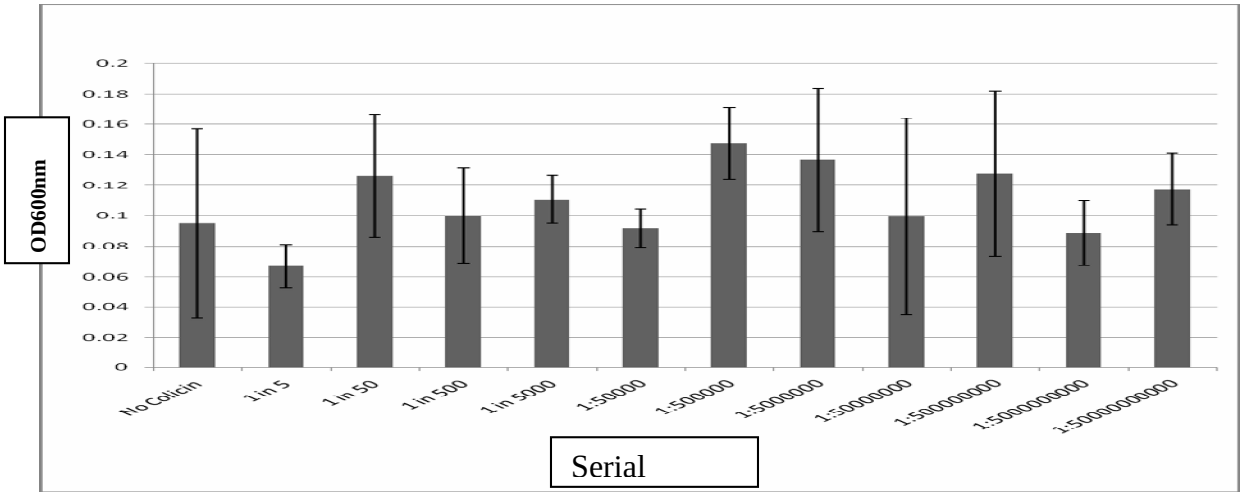


Figure.11 Backup test was carried out on biofilms grown at room temperature for 48h to confirm if colicin E1 treated biofilms cells grew or a fraction grew at room temperature. Result deduced shows that there is no significant biofilm growth present in the column that contained 1 in 5 dilution of col E1. This result also confirms that a fraction of biofilm growth were inhibited in dilutions 1 in 50 to 1 in 50000 which might be due to the after effect of the action of the colicin. Biofilm-attached pegs were placed onto a fresh 96-well microtiter plate containing 1: 5 serial dilution of the test colicin and incubated overnight (24h), at room temperature. The peg lid was then rinsed and placed onto a fresh 96-well microtiter plate, containing sterile LB broth media and incubated for another 24h. Cell growth was seen as turbidity in wells that harbours biofilms and measured at an absorbance of 600nm using a spectrophotometer.

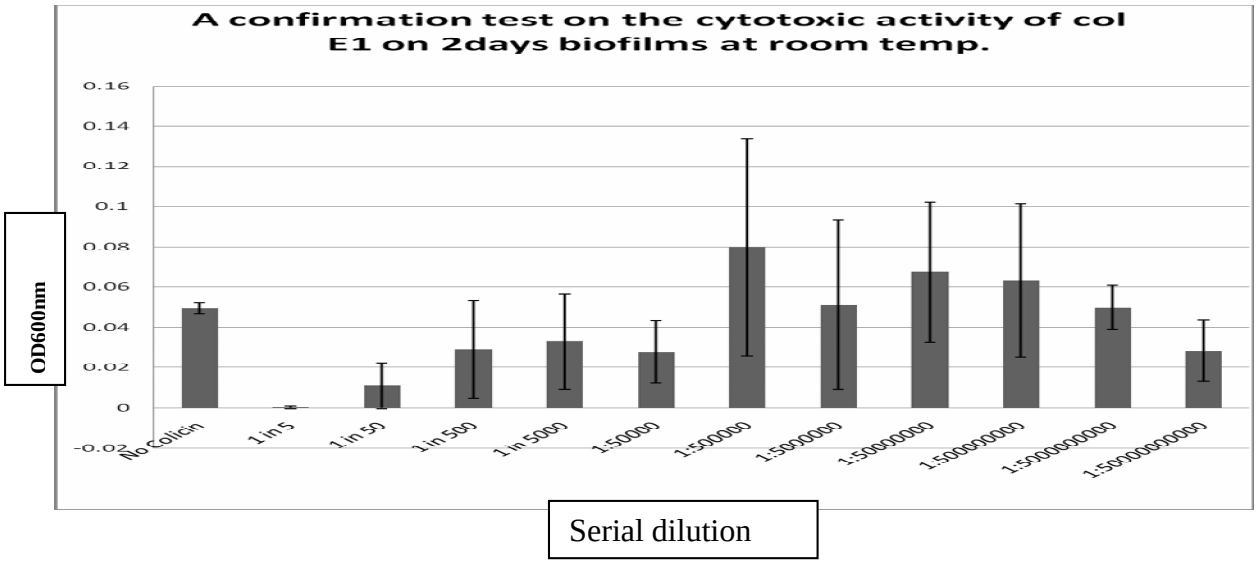


Figure.12 Backup test was done on biofilms grown at 37ºC for 48h to confirm if colicin E1 treated biofilms cells grew or a fraction grew at optimum temperature. Result shows that biofilms are resistant to col E1 at 37°C . The aftereffect of col E1 induced biofilm growth. Biofilm-attached pegs was placed onto a fresh 96-well microtiter plate containing 1: 5 serial dilution of the test colicin and incubated overnight (24h), at 37ºC. The peg lid was then rinsed and placed onto a fresh 96-well microtiter plate, containing sterile LB broth media and incubated for another 24h. Cell growth was seen as turbidity in wells that harbours biofilms and measured at an absorbance of 600nm using a spectrophotometer.

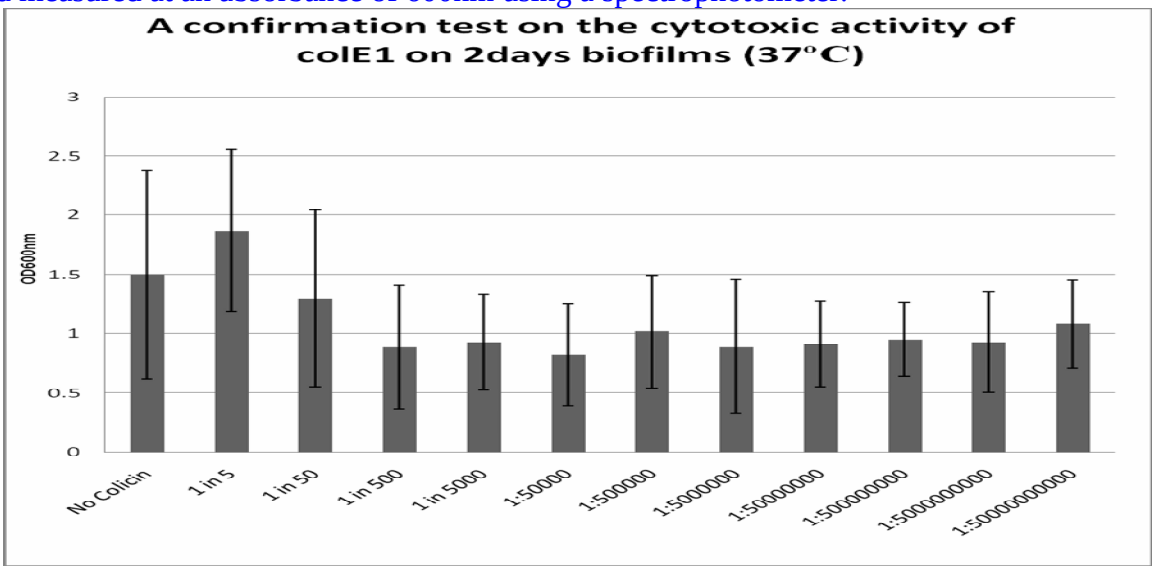


Figure.13 Backup test was performed on biofilms grown at ambient temperature for 48h to confirm if colicin E3 treated biofilms cells grew. Result clearly shows that biofilms are resistant to 1 in 5 fold dilution of colicin E3. Biofilm-attached pegs was placed onto a fresh 96-well microtiter plate containing 1: 5 serial dilution of the test colicin and incubated overnight (24h), at room temperature. The peg lid was then rinsed and placed onto a fresh 96-well microtiter plate, containing sterile LB broth media and incubated for another 24h. Cell growth was seen as turbidity in wells that harbours biofilms and measured at an absorbance of 600nm using a spectrophotometer.

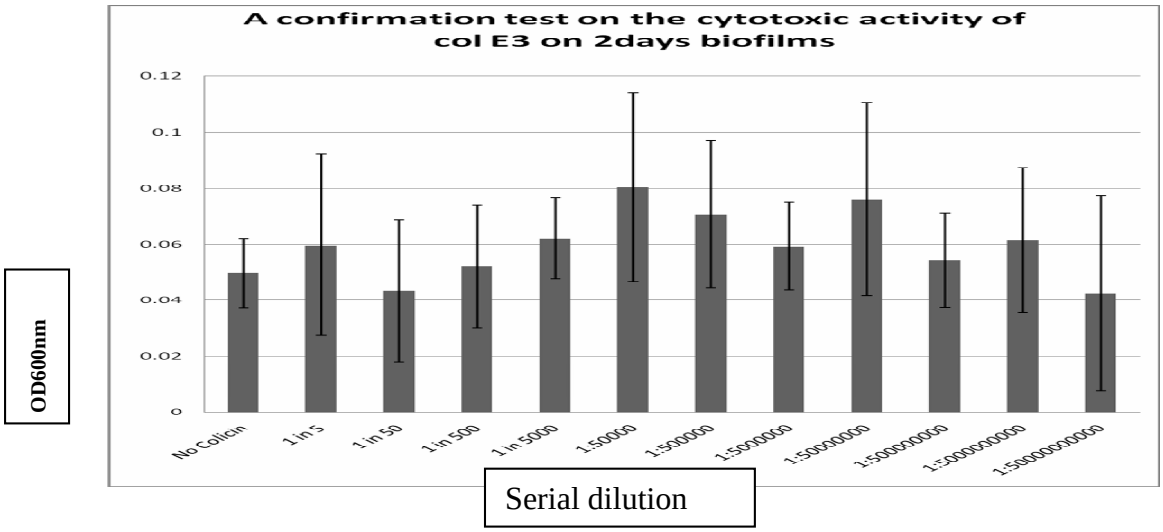
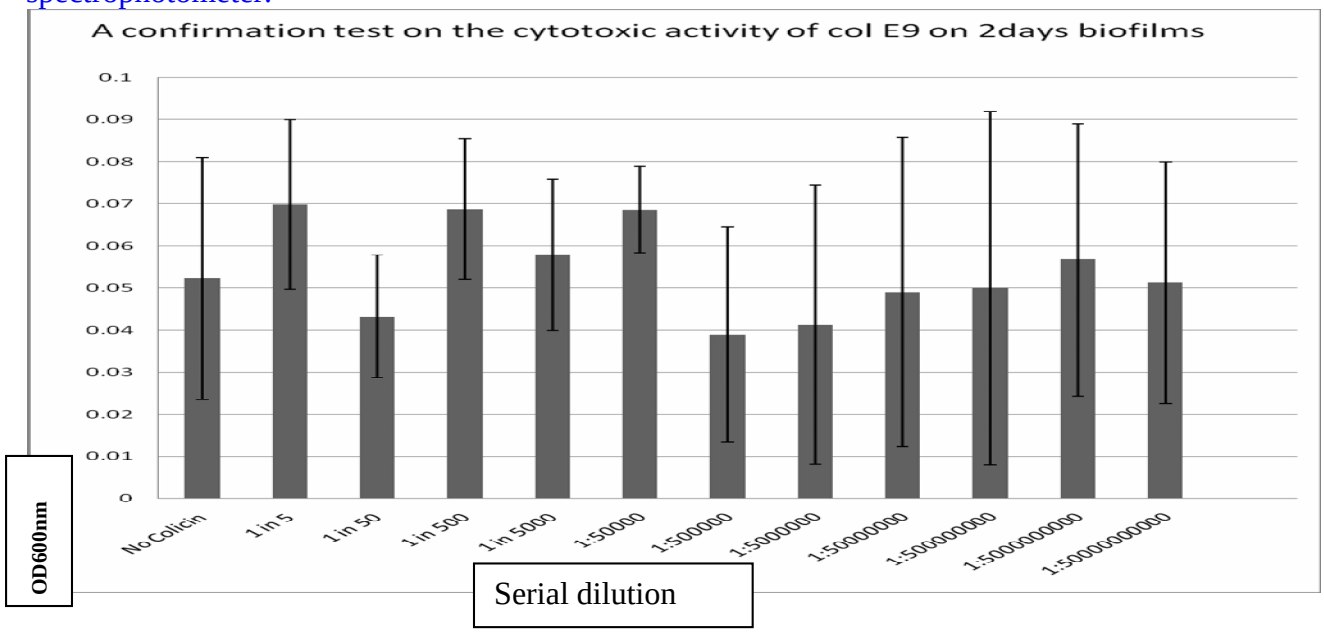


Figure.14 Backup test was performed on biofilms grown at ambient temperature for 48h to confirm if colicin E9 treated biofilms cells grew. Result clearly shows that biofilms are resistant to 1 in 5 fold dilution of colicin E9. Biofilm-attached pegs was placed onto a fresh 96-well microtiter plate containing 1: 5 serial dilution of the test colicin and incubated overnight (24h), at room temperature. The peg lid was then rinsed and placed onto a fresh 96-well titer plate, containing sterile LB broth media and incubated for another 24h. Cell growth was seen as turbidity in wells that harbours biofilms and measured at an absorbance of 600nm using a spectrophotometer.



This experiment indicates that growth condition serves as an important factor in biofilm resistance to killing by colicin. At room temperature on 2 day old biofilms, col E1 shows some cytotoxic activity in dilutions 1 in 5 to 1 in 500. Other colicins have different cytotoxic activities and so may have different effects on biofilms. To test this we treated biofilms with a ribonucleasecolicin E3 and an endonuclease colicin E9.

Results from other colicins shows no killings on biofilms and became more resistant to this toxins.

Viability of colicin treated biofilms

A second biofilm killing assay was developed in the laboratory in order to confirm that the first biofilm killing assay developed in the laboratory above had worked. Viability of colicin treated biofilms was done to determine if the colicins treated biofilms grew or a fraction of the biofilms grew.

Under sterile conditions biofilm-attached pegs were placed onto a fresh 96-well microtiter plate containing 1:5 serial dilution of the test colicin and incubated overnight (24h), at room and optimum (37ºC) temperature. After 24h, the peg lid was rinsed and placed onto a fresh 96-well titer plate, containing sterile LB broth media and incubated for another 24h. Cell growth was seen as turbidity in wells that harbours biofilms and was measured at an

absorbance of 600nm using a spectrophotometer. Figures 11, 12, 13 and 14 were backups conducted to confirm the result obtained from the first procedure.

The result obtained from this method agrees and is consistent with the previous method concluding that this method was a success.

In the presence of colicin, bacteria growth is either induced or inhibited as seen in the result section. The MIC on plate was determined and measured base on the activities of the antimicrobial proteins against planktonic bacteria. The MIC determined in this study serves as an important reference in the treatment of *E.coli*-related infections [Penfold *et al*., 2000). The minimal inhibitory concentration of colE1, E3 and E9 on plates are $1.2x10^{-2}$nM, $9.4x10^{-2}$nM and $7.5x10^{-2}$nM respectively. The cytotoxic actions of the colicins tested indicated that the MIC of the pore-forming colE1 is approximately 10times lower than the MIC of the enzymatic colicins. The reason why colE1 showed to be more powerful compared to colE3 and colE9 could be as a result of the mechanism of their cytotoxic domain action. The pore-forming colE1 kills cells by inserting and forming voltage-dependent ion channels inside the cytoplasm of the target which depolarizes the cytoplasmic membrane (Schwart and Helinski,1971). The cell killing potential of the pore forming colicin E1 is specific in that one molecule kills one cell [O'Toole *et al*., 2000]. They kill cells from a distance without crossing into the cytoplasmic membrane unlike the nuclease type colicin whose cytotoxic domain must pass into the cytoplasm where they act enzymatically on DNA, rRNA and tRNA [O'Toole *et al*., 2000]. The enzymatic colicincatalyzes the hydrolysis of phosphodiester bonds in the bacterial cytoplasm, thereby eliciting cell death [O'Toole *et al*., 2000]. ColE9 kills bacteria through non-specific degradation, magnesium-dependent cleavage of chromosomal DNA (Szomolay *et al*., 2005; Walker *et al*., 2002). ColE3 specifically cleaves 16S rRNA at the A site between A1493 and G1494, this occurs only in the context of the intact 70S ribosome (Walker *et al*., 2004) to arrest protein synthesis. Other studies including Ohno and Imahori, (1977,1978) reported that at high concentrations of the colicin E3, cleavage of both 30S subunits and naked 16S rRNA is done but with a lack of specificity.

The graphical representation of the result obtained from the cytotoxic activities of the colicins against *E. coli* BW25113 strain grown in the planktonic state (liquid media) shows that the cells reduce in number. The activity of each colicin was measured based on the MIC. From the result illustrated above, the pore-forming colicin is more active than the nuclease colicins. This is true for the reason being that the minimal inhibitory concentration of col E1 ($1.98x10^{-2}$nM) is approximately 10times less concentrated and more active than the minimal inhibitory concentration (0.2nM) for colicin E3 and 10.4 times less concentrated and more active than the MIC (0.6nM) for colicin E9. The efficiency of cell killing by DNasecolicin (col E9) has been reported by Walker *et al*., (2007) to be dependent on the level of anionic phospholipid in the target cell and the positive charge on the nuclease. This experiment was a success.

In the second phase of this experiment, the cytotoxic activity of colicins E1, E3 and E9 against *E.coli* grown in the biofilm state was performed in order to compare the efficiency in which these colicins kill cells that grow on agar plate, liquid cultures and

solid surfaces. The result analysed showed that 2 days culture of BW25113 strain grown as biofilms at ambient temperature were resistant to the cytotoxic action of colicin E3 and E9 but sensitive to colE1 with the minimal biofilm eradication concentration of 23.98μM. However, when this same strain was cultured as biofilms for 3 days at ambient temperature, resistance to colE1 developed. Stronger resistance was observed with biofilms cultured at an optimum temperature (37ºC) which may be as a result of stronger biofilm formation. This implies that biofilms are more powerful and much more established when grown at optimum or favourable conditions than in environmental condition (less favourable condition), as seen in the controls grown at optimum temperature compared to controls grown at ambient temperature. Kimberly Jefferson, (2004) reported that under favourable conditions or appealing environment like the human body, the bacteria switch to biofilm mode of growth and remain fixed. Previous studies show that in vitro biofilm formation by *E.coli* is subject to variability and is dependent on growth conditions (Ying *et al*., 2008). Kristi *et al*., (2006) reported that biofilms show inherent resistance to high levels of antimicrobial agents and this makes treatment of biofilm infections relatively difficult and costly.

Studies have shown that antimicrobial agents required to kill biofilms is 10-1000 times more concentration than the amount necessary to kill the same amount of free-swimming bacteria cells (Naoki *et al*., 2005; Ogawa *et al*., 1999; Ohno and Imahori, 1978). Sub-inhibitory concentrations of various antimicrobials have been reported by several researchers to both stimulate and impede biofilm formation (Zakharov and Cramer, 2002).

Aviability of colicin treated biofilms experiment was done to further confirm my findings. Colicin-treated biofilms cultured at room temperature for 72h and at optimum temperature for 48h grew rapidly. The reason for this might be that the biofilms grew more matured, established and fixed at this conditions and this might be the reason for the increase in resistance to colicin E1 of a 1 in 5 dilution concentration of 23.98μM. The verse difference in biofilm sensitivity to the cytotoxic activity of the colicin residue in each well could be as a result of the unstable temperature since the room temperature can fluctuate between 20ºC and 25ºC. However at stable temperature (37ºC) much difference was not observed in response to the activity of the antimicrobial proteins. However, using a higher concentration of colicinE1 against biofilms would be appositebefore concluding whether or not biofilms were completely resistance or sensitive to colE1 at the optimum temperature.

The method developed successfully showed that colicin E1, E3 and E9 were active against non-pathogenic *E.coli* in the planktonic state but not active in the biofilm state with a little exception to colE1 which was active at the condition indicated in the result section. Since this protocol is a success, it should be used as headway for *Pseudomonas eruginosa* which are pathogenic and produces pyocins which are bactericidal against strains of *Pseudomonas eruginosa*. This is important since drug resistant strains of *Pseudomonas eruginosa*has evolved.

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