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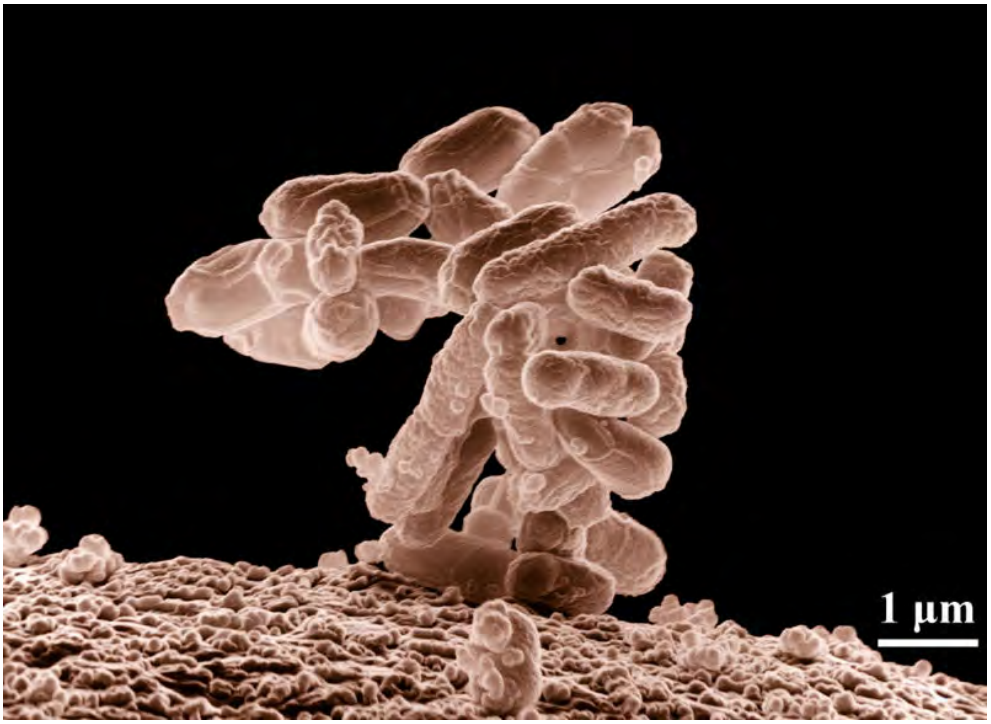
Effect of nitric oxide on biofilm formation by *Escherichia coli*



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SUMMARY

Bacterial biofilms are very complex and organized structures that are highly resistant to antibiotics and host defences. Biofilm is medically significant because of its formation on medical implants like catheters. Uropathogenic *E. coli* (UPEC), the most frequent cause of urinary tract infections, are associated with catheter-related biofilms. In this study, I investigated the effect of nitric oxide (NO), the molecule that is widely used as a signaling molecule in biological system, on biofilm formation. Two nitric oxide donors , Sodium Nitroprusside Dihydrate (SNP) and DETA NONOate (chemical name 1-[N-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate) were tested for effects on biofilm formation.

I found that pathogenic *E. coli* could form biofilms when only curli or both curli and cellulose are present on their outer surface. Curli fibers are adhesive surface fibers expressed by *E. coli* that bind several host extracellular matrix and were assumed to have a role in pathogenesis.
I found that NO had an inhibitory effect on biofilm formation by the strains that expressed both curli and cellulose on their outer surface. NO inhibited biofilm formation by *E. coli* K-12 bacterial strains carry a *flu* gene (encoded Antigen43 protein that is mainly involve to form biofilm in these strains) on a plasmid, whereas biofilms formation by control strains (Δ*flu*) was not effected by NO. Strains that expressed P fimbriae had a general ability to form biofilm but biofilm formation was not significantly affected by NO. Biofilm formation was inhibited in all cases by nanomolar and micromolar concentrations of DETA and by nanomolar concentrations of SNP. At millimolar concentration of DETA and micromolar concentration of SNP, biofilm formation by these strains was also inhibited; however, I found that bacterial growth also was effected at this concentration range. Moreover, at nanomolar and micromolar concentration of DETA and at nanomolar concentration of SNP, growth of these strains was not significantly effected. Further studies to be needed to pinpoint the concentration of DETA & SNP that acts against biofilm formation and to possible interaction of NO with other components of immune response before using NO as treatment against biofilm related infectious diseases.

Source of picture in cover page: http://en.wikipedia.org/wiki/Escherichia_coli

INTRODUCTION

Urinary tract infection

Urinary tract infections (UTI) are a serious health problem affecting millions of people throughout the world. UTI cause significant medical expenditure amounting to more than a billion dollars each year only in United States. Women are more vulnerable to UTI; 40 to 50% of all women have experienced a urinary tract infection at least once and many women experience recurrent infection. UTI now appear as more common disease for both women and men, especially in the perspective of urinary catheterization (Cegelski *et al*. 2008).

Uropathogenic *E.coli* (UPEC) are the most common causative agent of UTI. Pathogenic bacteria need to be able to remain at the infection site. Bacteria require specific proteins, called adhesins, to establish a successful interaction between bacteria and host that enable bacterial colonization. Usually all clinical UPEC isolates express type 1 fimbriae, a fibrillar structure that promotes bacterial adhesion to host receptors (Garofalo *et al.* 2007). As a consequence, UPEC binding to the target uroepithelial cells by type 1 fimbriae causes a bladder infection. This infection initiates a cascade to form a community of bacteria attached to the bladder wall. These organized bacteria cannot be flushed away by the flow of urine. Bacteria can remain attached for many days or weeks to the uroepithelial cells after the acute infection has been eliminated by standard antibiotic treatments. This might result in recurrent infection (Cegelski *et al.* 2008).

An important reason for treatment failure in UTI is the formation of catheter-related biofilm. If the patient keeps a urinary catheter for 30 days or longer, catheter-associated infections will commonly develop (Foxman 2002). UPEC bind to the catheter, replicate very rapidly and form a community, called biofilm, which helps to protect bacteria from the host defences in this community. These organized communities are highly resistant to antibiotics (Mah *et al*. 2001, Costerton *et al.* 1999).

Biofilms

Biofilms can be defined as communities of microorganisms attached to a surface. Differentiation of planktonic cells into a mature complex biofilm causes many phenotypic changes within the bacterial cells that give rise to increased resistance against antibacterial agents (Cegelski *et al.* 2008). Planktonic bacterial cells first reversibly attach to the solid surface in response to specific environmental cues, such as nutrient availability (O'Toole *et al.* 2000). The transition from reversible to irreversible attachment occurs through the production of extracellular polymers by the bacteria and/or by specific adhesins located on pili and fimbriae (Van Houdt *et al.*2005).

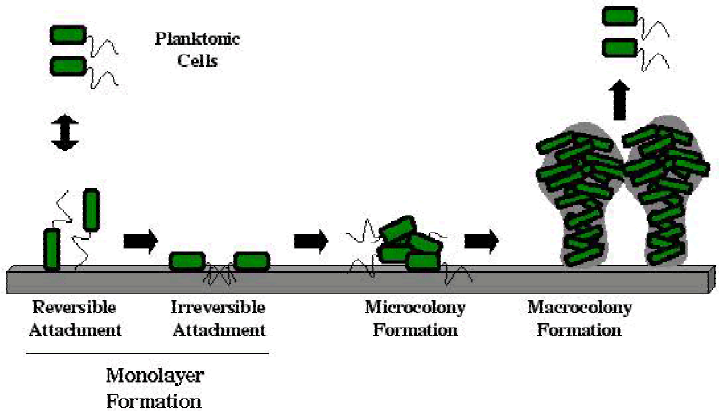


Figure 1: A model for biofilm formation in *Pseudomonas aeruginosa*. This picture is taken from the website (http://www.dartmouth.edu/~gotoole/papage.html) of O'Toole lab, Department of Microbiology and Immunology, Dartmouth Medical School, Hanover with permission from the copy-right owner.

Biofilm formation depends on the nature of the bacteria species as well as environmental conditions. Biofilm formation is thought to begin when bacteria sense environmental conditions that trigger the transition to life on a surface. Curli and cellulose are two major components in the outer surface of *E. coli* (Chirwa *et al.* 2003). The contribution of cellulose in virulence is yet to be confirmed but curli fimbriae (a proteinaceous cell surface structure) have a role in virulence-related features (Sjöbring *et al.*1994, Herwald *et al.* 1998). However, a common picture of the role of curli fimbriae in pathogenicity is still elusive, since *E. coli* does not consistently express curli fimbriae. Cellulose production confers bacterial cell–cell interactions, adhesion to abiotic surfaces (biofilm formation) (Zogaj *et al*. 2003). *E. coli* widely expressed cellulose in their outer surface as exopolysaccharide (Zogaj *et al*. 2003).

Type 1 and P fimbriae are two important adhesins found in most pathogenic *E. coli* and are involved in adhesion of UPEC to cells of the urinary tract and to catheters as well. They have different tip-associated adhesins (Connell *et al*. 1996, Söderhäll *et al.* 1997). P fimbriae are most strongly associated with UTI in clinical studies. P fimbriae *E. coli* contain the PapG adhesin that preferentially binds to globoside, a glycosphingolipid containing acetylated amino sugars and simple hexoses (GalNAcβ3Galα[1-4] GalβGlcβcer) that is abundant in the upper urinary tract of humans (Söderhäll *et al.* 1997). Type 1 fimbriae carry adhesins that mediate mannose-specific binding to receptors present on the human bladder epithelium by *fimH*. The role of the *E. coli* mannose-specific adhesin in virulence has been debated, but it may play a role in urinary tract infection (Connell *et al.* 1996).

The bacterial phase-variable surface-located adhesin antigen 43 (Ag43) is also associated with urovirulence. Ag43 (encoded by the *flu* gene) is a self-recognizing adhesin that confers characteristic surface properties on host cells, such as auto aggregation and frizzy colony morphology (Hasman *et al*. 2000). A large amount of Ag43 is expressed during bladder infection by uropathogenic *E. coli* and participates in biofilm formation by Ag43-Ag43 interaction (Anderson *et al*. 2003). Ag43 is expressed on the surface of UPEC cells located within intercellular biofilm-like bacterial pods in the bladder epithelium, indicating that it may contribute to survival and persistence during prolonged infection (Anderson *et al*. 2003)
Biofilm formation is medically significant for its widespread involvement in oral microbes on teeth and chronic *Pseudomonas aeruginosa* infections in the lungs of cystic fibrosis patients (Mah *et al*. 2001, Whiteley *et al.* 2001). Biofilms also form on medical implants other than catheters such as pacemakers and hip replacements etc (Cegelski *et al*. 2008). Therefore, biofilm-based treatment cost is increasing rapidly and it is now beyond billion dollars. It is

now a very costly and stressful procedure to replace the infected implant or fight the infection with antibiotics, the only treatment currently available against biofilms. Therefore, to reduce the cost of treatment is one of the major goals of scientist, at least those dealing with infectious medicine.

Minimum inhibitory concentration

In microbiology, minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that required to inhibit visible growth of microorganism in a media after a standard time of incubation. MIC are important in laboratories to confirm resistance of microorganisms to an antimicrobial agent. MIC usually tests the sensitivity of antimicrobial agents in their planktonic phase. MIC values can be determined by a number of standard test procedures. Serial dilutions are made of the antimicrobial agent in bacterial growth media, the most commonly used method. The test organisms are then added to the dilutions of the products, incubated, and scored for growth.

Nitric Oxide

The first defence against bacterial infection is the innate immune system. NO contributes to this early response by its bactericidal activity (Carlsson *et al.* 2005). NO is produced in large amounts by macrophages and neutrophils but also by epithelial cells. The generation of NO is due to the activity of an inducible NO synthases (iNOS) expressed by these cells. Many cell types can express iNOS for their function in host defense against microbial and viral pathogens (Bogdan 2001), leading to the formation of NO radicals or ONOO- in the host cell. The nitric oxide (NO) acts as an antibacterial agent (De Groote and Fang, 1995). It has been shown that NO interacts with the superoxide anion and produces the highly reactive peroxynitrite anion [further converted into reactive nitrogen intermediates (RNIs)], which are very potent antimicrobial agents (Frang 1997, Bogdan *et al.* 2000). RNI are potentially highly reactive molecules that can be produced continuously in the organisms as by-products of respiratory metabolism (Hassett *et al.* 2002) RNI can damage DNA, lipids, and proteins of the cells when the production of reactive oxygen intermediate (ROI) and/or RNI overwhelms the capacity of the cell to remove such molecules (Barraud *et al.* 2006).

From the several studies it has been shown that low levels of NO induce a transition from biofilm growth to the planktonic growth (Barraud *et al.* 2006)**.** NO is an effective inhibitor of respiration of bacteria and will bind to the metalloenzymes found in electron transport chains at low mM concentrations (Wink *et al.* 1998) NO is a highly diffusible molecule that acts as a broad signal molecule in biological system (Beckman *et al*. 1997). In addition, NO is the main precursor of peroxynitrite ($ONOO^{-}$) that can interact with proteins, lipids, carbohydrates and DNA. $ONOO^{-}$ can form nitrosoperoxocarbonate by reacting with carbon dioxide, which through oxidation and nitration mechanisms changes the structure and function of these compounds to lead to oxidative damage of tissues (Bloodsworth A. *et al.* 2000).

Aim

The aim of my experiments was to see the effect of NO on biofilm formation by different pathogenic *E. coli* strains. The growth of microorganisms was also observed in presence of similar NO concentration. I also studied the effect of NO on strains that expressed biofilm formation components (Ag43). I found some effect of NO on biofilm formation in pathogenic *E. coli* strains. Therefore, NO could be used to treat biofilm mediated diseases in future.

RESULTS

The bacteria carrying curli and/or cellulose and the bacteria carrying P fimbriae and type 1 fimbriae are all derived from the naturally occurring wild type strains. The gene or genes of these strains encoded these structures are chromosomally encoded in their native content. On the other hand, the bacteria that carry a plasmid encoded *flu* gene express this under the control of arabinose. This makes possible constitutive expression of the *flu* gene by adding arabinose in the growth medium.

Biofilm formation by different bacterial strains

Before testing the effect of NO on biofilm formation of the bacterial strains, I checked the general ability of these bacterial strains to form biofilm. All strains of *E. coli* that were used in my experiments could not form biofims (**Figure 2)**. The strain producing both curli and cellulose and the strain producing only curli formed more biofilm than strains lacking curli. Bacterial strains containing a plasmid carried *flu* gene as well as Δ*flu* strain could form biofilm. *E. coli* DS17 encoding wild type P fimbriae formed more biofilm than strains lacking functional fimbriae or encoding type 1 fimbriae.

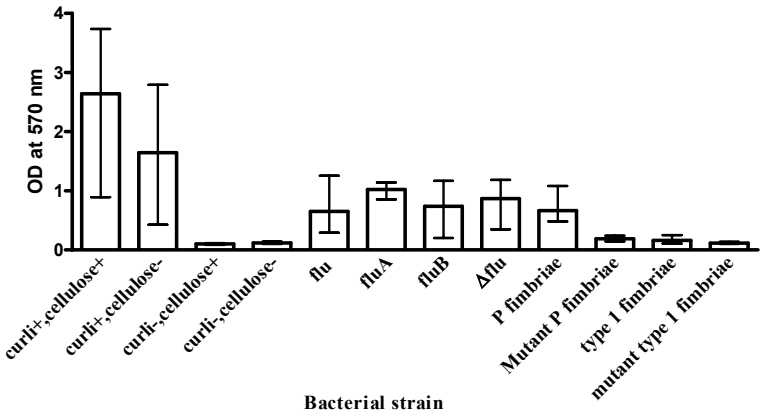


Figure 2: Biofilm formation by different bacterial strains. Bacterial cells were grown on a microtiter plate surface at 37ºC overnight. Biofilm formation was quantified by crystal violet staining. The data represent mean ± standard error of mean from three replicates. In the graph curli$^+$,cellulose$^+$(#12); curli$^+$,cellulose$^-$ (WE1); curli$^-$,cellulose$^+$ (WE11); curli$^-$, cellulose$^-$ (WE16); *flu* (MS1230); *fluA* (MS1232); *fluB* (MS1231); Δ*flu* (MS1199); P fimbriae (DS17); Mutant p fimbriae (DS17-8); type 1 fimbriae (NU14); mutant type 1 fimbriae (NU14-1).

Growth of bacteria in presence of NO

Before testing the growth of different strains in the presence of NO, the minimum inhibitory concentration (MIC) of NO for these strains needed to be determined. I used 102.4 mM of SNP to determined MIC value but I still found some growth of micoorganisms in the presence of this concentration of NO. Though I observed the growth of four different strains expressing curli and/or cellulose [curli$^+$,cellulose$^+$(#12); curli$^+$,cellulose$^-$ (WE1); curli$^-$,cellulose$^+$ (WE11); curli$^-$,cellulose$^-$ (WE16);], four different strains of *flu* gene [*flu*$^+$ (MS1230); *fluA* (MS1232); *fluB* (MS1231); Δ*flu* (MS1199);] and four different strains of wild or mutated form of P fimbriae and type 1 fimbriae, here I present only the growth of one strain represent each group (Figure 2). The growth of other strains in the same group (containing the same molecule in their surface structure) was almost similar (data not shown here). The growth of

different strains did not vary significantly in the presence of different lower concentrations, in the micromolar and nanomolar ranges, of DETA and SNP **(Figure 3).** Above 100 µM DETA or 15 µM SNP, the growth of all bacteria in this study declined. However, no MIC values could be determined. Growth of bacteria containing type 1 fimbriae was different in the presence of SNP. Exceptionally from others, the growth increased with increasing concentration of SNP and then reduced gradually at later concentration.

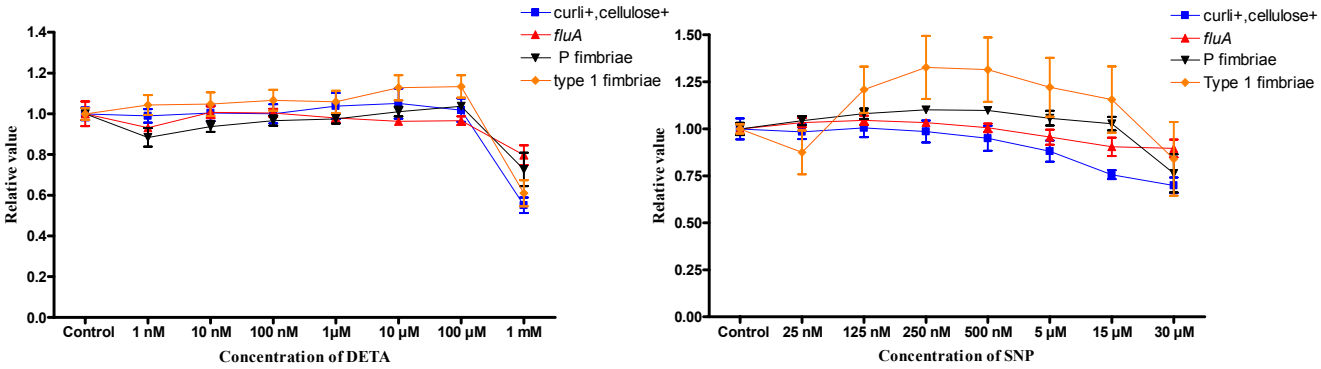


Figure 3: Growth of different strains in the presence of SNP and DETA. Bacteria were grown in LB with inhibitors as shown below the panels for 24 hours on 8-well strips, starting with 10^6 colony-forming units (CFU) per ml. Relative numbers of cells were estimated using alarmarBlue, a redox dye that quantifies metabolic reactions, and normalized to the value obtained in cultures lacking inhibitors. (a) Growth in presence of DETA, (b)Growth in presence of SNP. The data represent mean ± standard error of mean from three replicates. In this graph curli$^+$,cellulose$^+$ (#12); *fluA* (MS1232); P fimbriae (DS17); type 1 fimbriae (NU14).

Effect of NO on biofilm formation by strains in the presence of curli and cellulose

To determine the effect of NO on biofilm formation by strains expressing curli and/or cellulose on their outer surface, different subinhibitory concentrations of NO donors was used. These concentrations did not effect the growth of the bacteria. On the other hand, I found an effect on biofilm formation **(Figure 4a)** between 10 nM and 1 µM, significantly arround 100 nM concentration of DETA. It is really hard to pinpoint the cause of the fluctuation of biofilm inhibition below and above 100 nM concentration but I always got the same result for 100 nM concentration of DETA, around 25% inhibition of biofilm formation at this point. In the case of SNP the concentration range to inhibit biofilm formation was between 125 nM and 500 nM and biofilm formation was significantly inhibited arround 250 nM. . It was found that this concentration of NO donor had effect on biofilm formation only when the bacterial strain expressed both curli and cellulose but had no effect when only curli was present (WE1 strain). The other two strains WE11 (curli$^-$,cellulose$^+$) and WE16 (curli$^-$,cellulose$^-$) could not form biofilm (**Figure 2**). Biofilm formation of both curli$^+$,cellulose$^+$ (#12) and curli$^+$,cellulose$^-$ (WE1) strains in absence of DETA and SNP were measured and normalized it to 1 and used as reference value. Therefore, biofilm formation by both bacterial strain in presence of DETA and SNP were then compared with reference value. I presented all figures regarding biofilm formation and effect of NO donor on it in the same way. There were also significant effects on biofilm formation at 1 mM of DETA and 30 µM of SNP but this concentration also affected the growth of the bacteria (**Figure 3**).

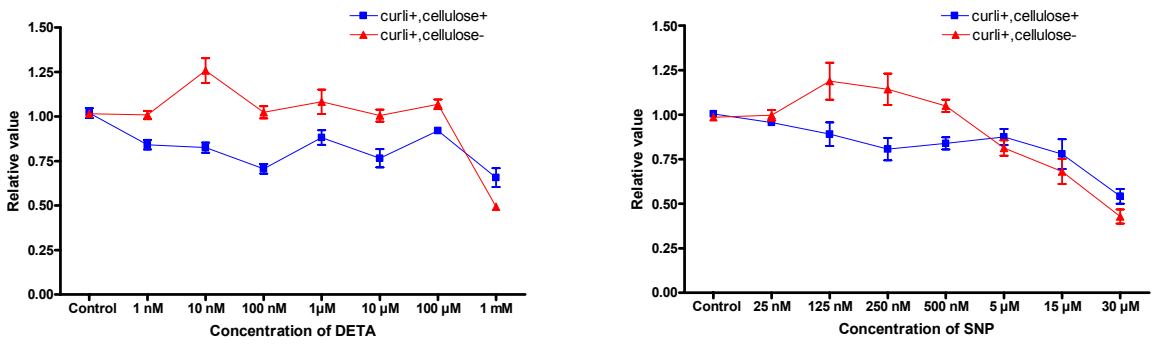


Figure 4: Effect of DETA and SNP on biofilm formation. (a) Effect of DETA on biofilm formation by wild type strain. (b) Effect of SNP on biofilm formation by wild type strain curli$^+$, cellulose$^+$ (#12) and curli$^+$, cellulose$^-$(WE1). Biofilm formation was measured by the crystal violet assay method. Biofilm formation was compared to the biofilm formation by the control where no NO donor was added. The data represent mean ± standard error of mean from three replicates.

Effect of NO on biofilm formation by strains expressing plasmid-encoded *flu* genes

To confirm the role of Ag43 (product of the *flu* gene) in biofilm formation, the effect of NO on biofilm formation by *flu* gene was measured. The strains carrying *flu* gene were grown in the presence of arabinose yielding overexpression of the plasmid encoded Ag43 protein. Biofilm inhibition was observed as before by 10 nM to 1 µM concentrations of DETA for the *fluA* strain but not for the Δ*flu* expressed strain **(Figure 5a)**. The biofilm formation was reduced drastically above 100 µM DETA for MS1232 (*fluA*) strain and was reduced at the same concentration ranges for the MS1199 (Δ*flu*) strain. The biofilm formation was reduced significantly for both strains between 250 nM and 30 µM SNP **(Figure 5b)**. The other two strains MS1231 (*fluB*) and MS1230 (*flu*$^+$) also showed similar effects of NO on inhibition of biofilm formation (not shown in the figure 5) at the same concentration range of DETA and SNP.

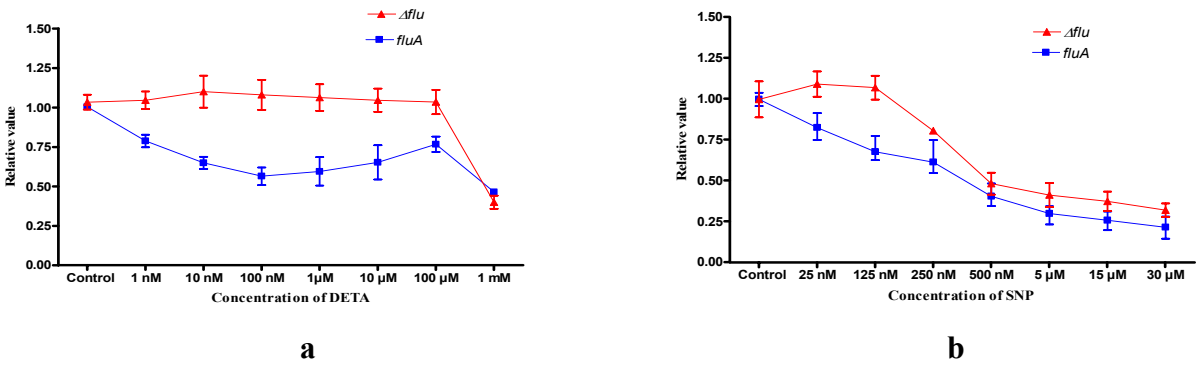
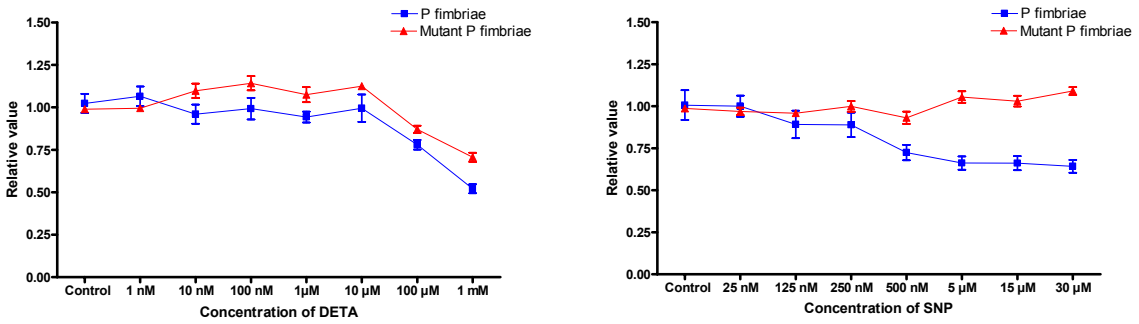


Figure 5: Effect of NO on biofilm formation by strains MS1232 plasmid encoded *(fluA)* and MS1199 (Δ*flu*) (a) Effect of DETA on biofilm formation by plasmid encoded *fluA* gene (strain MS1232) and plasmid encoded Δ*flu* gene (strain MS1199). (b) Effect of SNP on biofilm formation by plasmid encoded *fluA* gene (strain MS1232) and plasmid encoded Δ*flu* gene (strain MS1199). Both strains were grown in the presence of arabinose. Biofilm formation was measured by the crystal violet assay method. Biofilm formation was compared to the biofilm formation by the control where no NO donor was added. The data represent mean ± standard error of mean from three replicates.

Effect on Biofilm formation by strain carrying P fimbriae

To show the effect of NO on biofilm formation of the strain carrying P fimbriae, the biofilm formation of both wild type (DS17) and mutant (DS17-8) strains was observed in the presence of DETA (**Figure 6a**) and SNP (**Figure 6b**). For strain DS17, the inhibition of biofilm formation was not significant between 1 nM and 10 µM of DETA but biofilm formation was reduced at higher concentration than 10 µM (**Figure 6a**). The effect of DETA on biofilm formation by DS17 was almost similar with biofilm formation of DS17-8 strain. In presence of SNP, biofilm formation by DS17 was inhibited between 250 nm and 30 µM but the biofilm formation was not inhibited for the mutant strain (DS17-8) at these concentrations (**Figure 6b**).



a **b**

Figure 6: Efffect of NO on Biofilm formation by wild (DS17) and mutant (DS17-8) type P fimbriated strains. (a) The inhibitory effect of DETA on biofilm formation by P fimbriated bacterial strains. (b) The inhibitory effect of SNP on biofilm formation by P fimbriated bacterial strains. Biofilm formation was measured by the crystal violet assay method. Biofilm formation was compared to the biofilm formation by the control where no NO donor was added. The data were calculated as for previous figures and represent mean ± standard error of mean from three replicates. In this graph P fimbriae (strain DS17) and mutant P fimbriae (strain DS17-8).

DISCUSSION

Biofilm formation in presence or absence of curli and cellulose

The formation of biofilms by pathogenic bacterial strains has attracted the attention of many scientists recently due to its tremendous effect in different diseases and ubiquitous environments. *E. coli* expresses different adhesin like type 1, P fimbriae, curli etc. in their outer surface through which it has general ability to form biofilm. *E. coli* can form biofilm significantly in presence of both curli and cellulose **(Figure 2)**. Biofilm formation was reduced when curli was not present. It has been shown in *Salmonella* that overproducing of curli and cellulose by mutant bacteria can result in denser biofilms **(**Jonas *et al.* 2007**).** Surprisingly, I found repeatedly that biofilm formation was increased in WE1 (curli$^+$ and cellulose$^-$) strains at certain subinhibitory concentrations of NO (**Figure 4**). The reason behind this induction is not clear

Biofilm formation in presence or absence of Ag43 protein

Expression of the *flu* gene encoding the Ag43 protein that mediates cell-cell aggregation is one of the mechanisms to form biofilm, and UPEC in the bladder promotes biofilm formation by this mechanism (Ulett *et al*. 2007). The control strain MS1199 (Δ*flu)* could make significant amounts of biofilm, but the biofilm formation of this strain was not affected in the presence of DETA (**Figure 5a**). There might be other components in strain MS1199 that made biofilms that are not effected by DETA. DETA inhibited biofilm formation by the MS1232 strain that expressed plasmid encoded Ag43A but did not inhibited the MS1199 (Δ*flu)* strain. I can conclude that Ag43A might have a role in biofilm formation that is inhibited by NO. Biofilm formation was also inhibited in presence of SNP between 250 nM and 30 μM (**Figure 5b**). But at the same time I found inhibition of bacterial growth at these concentrations of SNP (**Figure 3b**). By observing the effect of NO on strains that carry *flu*$^+$ gene, there might have role of SNP and DETA to inhibit biofilm formation by inhibiting Ag43 (product of *flu* gene). But it is yet to be known that how NO inhibit biofilm formation either by inhibiting Ag43 protein or by inhibiting *flu*$^+$ gene. So it can be assumed that Ag43 associated with biofilm development. However, it has been shown that Ag43 is dispensable in biofilm formation as it can be replaced by conjugative pili, alternative factors to form biofilm **(**Ghigo *et al.* 2001 and Reisner *et al.* 2003). Since there was no direct selection for the plasmid during my experiment, some cells might have been plasmid-free

The mechanism is not yet fully understood by which NO mediate inhibition of biofilm formation. Further research in clinical microbiology Department of KI will be conducted to assess the influence of nitric oxide on the immune system.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Three different uropathogenic *E. coli* and different mutants of them were used in this study. All wild type strains were clinical isolates. The relevant characteristics of bacterial strains used in this study are listed in Table 1.

Table 1: Bacterial strains

E. coli Strains	Parental strain	Relevant characteristics	Reference
# 12	Wild type	Wild type (curli$^+$, cellulose$^+$)	Chromek *et al.* 2006
WE 1	#12	Mutant (curli$^+$, cellulose$^-$)	Chromek *et al.* 2006
WE 11	#12	Mutant (curli$^-$, cellulose$^+$)	Chromek *et al.* 2006
WE 16	#12	Mutant (curli$^-$, cellulose$^-$)	Chromek *et al.* 2006
DS 17	Wild type	Wild type (expresses P-fimbriae)	Möllby *et al.* 1997
DS17-8	DS 17	Mutant (lacking functional adhesin)	Möllby *et al.* 1997
NU 14	Wild type	Wild type (expresses type 1 fimbriae)	Möllby *et al.* 1997
NU14-1	NU14	Mutant (lacking functional adhesin)	Möllby *et al.* 1997
MS1230	MG1655	pCO2 in OS56*, Amp^r Km^r	Ulett *et al.* 2007
MS1231	MG1655	pCO3 in OS56*, Amp^r Km^r	Ulett *et al.* 2007
MS1232	MG1655	pCO4 in OS56*, Amp^{r}Km^r	Ulett *et al.* 2007
MS1199	MG1655	pBAD/MycHisA in OS56*	Ulett *et al.* 2007
*OS56		MG1655 Δ*flu* Gfp$^+$ Amp^r	Ulett *et al.* 2007

Table 2: Plasmids

Plasmids	Relevant properties	Reference
pCO2	*flu* gene from MG1655 in pBADMycHisA-kan, Amp^r Km^r	Ulett *et al.* 2007
pCO3	*fluB* gene (c1273) from CFT073* in pBADMycHisA-kan, Amp^r Km^r	Ulett *et al.* 2007
pCO4	*fluA* gene (c3655) from CFT073* in pBADMycHisA-kan, Amp^r Km^r	Ulett *et al.* 2007

* Wild-type UPEC isolate

The MS1230, MS1231 and MS1232 strains carried the *flu*$^+$, *fluA* and *fluB* genes under the *araBAD* promoter on the plasmids pCO2, pCO3 and pCO4 respectively (**Table 2**). Bacteria except MS1230, MS1231, MS1232 and MS1199 were grown on blood agar plates (plates were supplied from the clinical microbiology division of Karolinska Hospital) over night. The bacterial strains MS1230, MS1231, MS1232 and MS1199 were grown in 10 ml Luria-Bertani (LB) broth supplemented with 100 mg/L ampicillin and 0.02 % arabinose overnight at 37°C with continuous shaking. All these strains were grown in the presence of arabinose yielding overexpression of the plasmid encoded Ag43 protein and its variants. LB broth was prepared by mixing 10g tryptone, 5g yeast extract, 10g NaCl in ~800mL distilled water and adjusted pH to ~7.3-7.5 and made volume to 1 L, then autoclaved. For biofilm experiments, all bacterial strains except MS1230, MS1231, MS1232 and MS1199 were grown on LB plates without NaCl to promote biofilm formation for 24 hours at 37°C. LB plates were prepared by mixing 10 g Bacto-tryptone, 5g yeast extract and 15 g agarose in ~800mL deionized (DI) H20, adjust pH to 7.5 and made volume to 1L and then autoclaved to sterilized, cool to 55 degree and pour into sterile petridish. Both LB plate and LB medium were prepared by lab assistant.

For biofilm experiments, the strains MS1230, MS1231, MS1232 and MS1199 were grown in LB broth without NaCl, supplemented with 100 mg/L ampicillin and 0.02 % arabinose for 24 hours at 37°C without shaking. All bacteria were stored at -70°C.

Nitric oxide donor

DETA NONOate (1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate, Alexis Biochemicals) and **S**odium **N**itroprusside **D**ihydrate (SNP, Sigma-Aldrich) stock solutions (2 mM for DETA and 30 µM for SNP) were prepared by dissolving the compound in PBS . PBS was made by dissolving 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 in 800ml distilled H_2O. Adjusted pH to 7.4 and made volume to 1L with additional distilled H_2O, then sterilized by autoclaving. Different dilutions were then prepared in LB. All SNP solutions were protected from light due to their light sensitivity and prepared freshly for each experiment.

Bacterial growth experiment (alamarBlue™ Assay)

The growth of bacteria in the presence of nitric oxide was measured by alamarBlue™ Assay. Bacterial colonies were resuspended from blood agar plates in PBS and adjusted to OD_{600} approx. 0.125 (10^8 colony forming unit/ml). Bacteria were then pelleted by centrifugation at 3500 g for 10 minutes and subsequently resuspended by pipetteing. This bacterial suspension was added to LB containing alamarBlue (1:10) [TREK Diagnostic Systems, Inc., USA] to a final concentration of 10^6 CFU/mL. The concentration of SNP (30µM, 15 µM, 5 µM, 500 nM, 250 nM, 125 nM and 25 nM) and DETA NONOate (1 mM, 100 µM, 10 µM, 1 µM, 100 nM, 10 nM and 1 nM) was prepared separately in LB during growth experiment. One hundred microliter of LB broth containing bacteria (10^6 CFU/mL), alamarBlue (1:10) and SNP or DETA in different concentrations were distributed on 8-well strips (Nunc-Immuno™, Denmark). Growth was monitored by measuring the OD at 570 nm at various time points. AlamarBlue consists of an oxidation-reduction (REDOX) indicator that yields a colorimetric change and a fluorescent signal in a response to a metabolic activity. Its reduction by the bacteria, the redox reaction, was measured by absorbance readings 570 nm at various time intervals

Minimal inhibitory concentration determination

Bacterial colonies were resuspended from blood agar plates in PBS and adjusted to OD_{600} approx. 0.125 (10^8 CFU/ml). This bacterial suspension was added to LB broth to a final concentration of 10^6 CFU/mL. Two hundred microliter of LB broth without salt containing bacteria (10^6 CFU/mL) and SNP in different concentrations were distributed on 96 well microtiter (Corning Incorporated, USA) plates and incubated statically at 37°C for 24 hours. I used SNP concentration starting from 30 µM to 102.4 mM gradually and observed the growth of bacteria after 24 hours with the naked eye.

Measurement of biofilm formation

To study biofilm formation in the presence of nitric oxide, bacterial suspension and different concentration of nitric oxide donor were prepared as described as above (final concentration of bacteria 10^6 CFU/ml). Two hundred microliter of LB broth without salt containing bacteria (10^6 CFU/mL) and SNP or DETA in different concentrations were distributed on 96 well microtiter plates and incubated statically at 37°C for 24 hours. After 24-h incubation, the

medium was gently removed and the microtiter plate wells were washed three times with 200 µl of PBS buffer using a multichannel pipette, and allowed to dry for 15 min.

The microtiter plate wells were subsequently stained with 200 µl crystal violet (0.3 % crystal violet in isopropanol ; BENEX Limited, Ireland) for 5 minutes. Unbound excess of stain was removed by washing the wells washed with tap water five times. The crystal violet bound to the exopolysaccharide in the biofilm was solubilized by the addition of 200 µl of ethanol (95%) per well and shaking on a horizontal shaker (500 rpm) for 5 min. The absorbance was measured at 570 nm. In all experiments, absorbance was measured with a plate reader, using the Revealation 4.21 program (Dynex technology, the microtiter company).

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