Characterization of functional amyloid curli in biofilm formation of an environmental isolate Enterobacter cloacae SBP-8

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Abstract

The biofilm formation by bacteria is a complex process that is strongly mediated by various genetic and environmental factors. Biofilms contribute to disease infestation, especially in chronic infections. It is, therefore important to understand the factors affecting biofilm formation. This study reports the role of a functional amyloid curli in biofilm formation at various abiotic surfaces, including medical devices, by an environmental isolate of *Enterobacter cloacae* (SBP-8). A knockout mutant of *csgA*, a structural gene of curli, was created to study the effect of curli on biofilm formation by *E. cloacae* SBP-8. Our findings confirm curli production at 25°C and 37°C in the wild-type strain. We further investigated the role of curli in the attachment of *E. cloacae* SBP-8 to glass, enteral feeding tube, and foley latex catheter. Contrary to the previous studies reporting the curli production below 30°C in most biofilm-forming bacterial species, we observed its production in *E. cloacae* SBP-8 at 37°C. The formation of more intense biofilm in wild-type strain on various surfaces compared to curli-deficient strain (Δ*csgA*) at both 25°C and 37°C suggested a prominent role of curli in biofilm formation. Further, electron and confocal microscopy studies demonstrated the formation of diffused monolayers of microbial cells on the abiotic surfaces by Δ*csgA* strain as compared to the thick biofilm by respective wild-type strain, indicating the involvement of curli in biofilm formation by *E. cloacae* SBP-8. Overall, our findings provide insight into biofilm formation mediated by curli in *E. cloacae* SBP-8. Further, we show that it can be expressed at a physiological temperature on all surfaces, thereby indicating the potential role of curli in the pathogenesis.

Introduction

Biofilm is regarded as a complex, sessile community of microbes found either attached to a surface or buried firmly in an extracellular matrix (ECM) as aggregates (Roy et al., 2018). The biofilm lifestyle provides the bacteria strong ability to withstand adverse environmental conditions like starvation and desiccation. The biofilm also leads to a broad range of chronic diseases and contributes to a major cause of persistent nosocomial infections in immune-compromised patients (Singh et al., 2000; Davies, 2003). Bacterial colonization of abiotic materials and biofilm formation have serious detrimental consequences in hospital settings (Le Thi et al., 2001). Approximately 50% of nosocomial infections result from indwelling devices used for medical treatments, such as catheters, cardiac pacemakers, joint prostheses, dentures, prosthetic heart valves, and contact lenses (Piozzi et al., 2004; Wu et al., 2015). These implants provide an ideal surface for the attachment of bacterial cells. The biofilm-associated infections are mainly caused by opportunistic bacteria such as *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species from the ESKAPE group, many of which are resistant to commonly used antibiotics (Santajit and Indrawattana, 2016).

E. cloacae is the most commonly isolated species of the genus *Enterobacter*, which has been accepted as the etiologic agent of many infections in hospitalized and immune-compromised patients. Recently, it has been regarded as a significant bacterial pathogen (Zhang et al., 2016). *E. cloacae* is often isolated from nosocomial infections, including pneumonia, urinary tract, and bloodstream infections. It is responsible

for 3 to 6% of bloodstream infections, with approximate mortality rates ranging from 27 to 61% (Nyenje et al., 2013). Nosocomial infections related to the use of medical devices are associated with a high risk of mortality and increased economic costs (Costerton et al., 1987). It is important to understand the factors that promote bacterial adhesion and the formation of multicellular communities on abiotic surfaces to determine how medical, industrial, and ecological biofilm contamination may be prevented. The formation of biofilms in both pathogenic and non-pathogenic strains requires time-dependent differential expression of ECM components (Flemming and Wingender, 2010). These key components facilitate a transition from an independent planktonic state to an organized multicellular community deeply engrained in the matrix (Costerton et al., 1995). To persist as a successful biofilm on any surface, the first step is the attachment to a surface. *Enteric* pathogens produce an array of adhesive structures and proteins for colonization on various biotic and abiotic surfaces (McWilliams and Torres). Various adhesive factors include curli fimbriae, flagella, cellulose, LPS, colanic acid, and several outer membrane proteins (Carter et al., 2016).

Amongst all the above-mentioned factors, curli fibres are regarded as one of the key factors which play a pivotal role in mediating biofilm formation and pathogenesis (Barnhart and Chapman, 2006). Curli are defined as functional amyloid secreted into the ECM of biofilm by both commensal and pathogenic members of *Enterobacteriaceae* (Zogaj et al., 2003; Kikuchi et al., 2005). Curli fibres are produced by a dedicated secretion pathway known as the nucleation-precipitation mechanism or the type VIII secretion system (Barnhart and Chapman, 2006). Seven curli-specific genes (*csg*) make up the structural components and assembly apparatus of the curli fibers, which are encoded by two divergently transcribed operons (*csgBAC* and *csgDEFG*) (Evans and Chapman, 2014). The curli subunit *csgA* is responsible for interaction with the biotic as well as the abiotic components. Curli are majorly involved in bacterial adherence to surfaces, cell accumulation and are a significant part of the ECM essential for establishing developed biofilms (Kikuchi et al., 2005). Curli display direct interaction with the substratum and form inter-bacterial bundles, allowing a cohesive and stable association of cells in biofilm (Prigent-Combaret et al., 2000). They are also regarded as a virulent element as they interact with a wide range of host proteins, such as matrix proteins and contact-phase proteins, which are suggested to help bacterial spreading in the host (Gophna et al., 2001). The expression of curli is responsive to many environmental factors such as temperature below 30°C, which strongly portrays the ecological role of this unique functional amyloid in non-host environments. Indeed, curli are implicated in the attachment to, or biofilm formation of, enteric pathogens on abiotic surfaces (Ryu et al., 2004; Goulter et al., 2010). To date, curli have primarily been studied in *Salmonella* and *Escherichia coli* biofilms (Van Gerven et al., 2018), where they serve as adhesive and structured support of the biofilm ECM, together with cellulose and extracellular DNA. However, the role of curli in the biofilm formation of *E. cloacae*, an important nosocomial enteric pathogen, is poorly explored.

The involvement of curli in the biofilm formation by *E. cloacae*, an eminent member of the ESKAPE group of organisms, is still elusive. Therefore, the present work aims to investigate curli's role in biofilm formation at different abiotic surfaces including commonly used medical devices such as catheters and feeding tubes, which are directly attached to patients for treatment. We also investigated curli-mediated

biofilm formation by *E. cloacae* at 25°C (room temperature) and 37°C (physiological temperature). As the environmental isolates can *en route* to hospital set-up via personnel and visitors contributing to hospital-associated infections, we used an environmental isolate, *E. cloacae* SBP-8, known to have pathogenic potential (Khan et al., 2020) as a test organism instead of a clinical isolate in the present study.

Materials And Methods

Bacterial growth and conditions:

We used *E. cloacae* SBP-8 (Accession No. NAIMCC-B-02025), an environmental isolate obtained from rhizosphere soil (Singh et al., 2017). The culture was grown in Luria Bertani (LB) broth media (Hi-Media) at 37°C with agitation (150 rpm) as and when required. As stated above, the mutant strain (ΔcsgA) was grown in LB broth supplemented with chloramphenicol (30µg/ml). To determine growth kinetics, the overnight grown culture of wild type and mutant strain (Δ*csgA*) were diluted 1:100 in YESCA media in a flask. The bacterial growth was monitored every hour at OD_{600} using a multiscan reader (Thermo-scientific). The glycerol stocks of both strains were made with 20% glycerol (SRL) and stored at -80°C for further use.

Generation of the major curli subunit csg **A knockout**:

We generated an in-frame deletion of *csgA* encoding the curli major sub-subunit of the curli operon of the *E. cloacae* SBP-8 genome, as described in (Sawitzke et al., 2013) with minor modifications. This method involves replacing the desired gene with an antibiotic (chloramphenicol) resistance cassette using the bacteriophage lambda red recombinase system. Electrocompetent cells were prepared by the standard protocol where bacterial cells were grown with the addition of 1M Arabinose till OD_{600nm} of 0.3–0.4, followed by washes with 10% glycerol in ice-cold conditions (Huang et al., 2014). The chloramphenicol (Cm^R) cassette was amplified from the pKD3 plasmid using specific primers. The amplicon contained chloramphenicol cassette flanked with sequences upstream (38 bp) and downstream (21 bp) of *csg*A. The purified Cm^R cassette (850 ng) was electro-transformed into *E. cloacae* SBP-8, having pACBSR (a plasmid consisting of λ-Red recombination system), using a 2 mm diameter Bio-Rad cuvette using gene pulsar II (Bio-Rad, USA) at 2.5 kV, 25 W for 5 ms. The transformants of *E. cloacae* SBP-8 with *csgA* deletion were grown on an LB plate supplemented with chloramphenicol and ampicillin at 37°C, followed by their screening by PCR using (Cm^R) cassette-specific confirmatory primers. The deletion of the gene was confirmed by using PCR amplification of the genes and the inserted antibiotic cassette. The primers used for the knockout generation and confirmation are listed in (**Supplementary material: Table 1**).

Congo Red Assay:

As previously described, the curli production by E. cloacae SBP-8 was determined by Congo Red assay (Zhou et al., 2013). Briefly, Congo Red agar plates were made by preparing yeast extract and Casamino

acid agar (YESCA: 1 g L^{-1} yeast extract, 10 g L^{-1} casamino acids, 20 g L^{-1} agar). After autoclaving, filter sterilized Congo Red (25 µg ml^{-1} final concentration; Sigma) and filter sterilized Brilliant Blue G (10 µg ml^{-1} final concentration; Sigma) were added. *E. cloacae* SBP-8 strains (wild type and Δ*csgA* mutant) were grown in LB broth and incubated at 37°C overnight. Five microliters of the overnight culture of each strain were spotted on the centre of a thick Congo Red agar plate. The plates were incubated at 25°C and 37°C for 24 to 96 hrs. Chloramphenicol (30 g ml^{-1}) was added to the growth media for the mutant strain. The images of the CR assay were captured using Oneplus 7 phone camera.

Evaluation And Quantification Of Biofilm Formation On Various Surfaces:

To evaluate the role of curli in biofilm formation by *E. cloacae* SBP-8, we compared the extent of biofilm formation in wild-type of *E. cloacae* SBP-8 with its mutant (Δ*csgA*) counterpart at 25°C and 37°C on different abiotic surfaces using the standard crystal violet assay as described in (Djeribi et al., 2012). We used the Enteral feeding tube (Romolene batch no: 171122361, India) and latex catheter (Romolene batch no: G20082297, India) as the medical devices. Culture tubes and glass slides (Borosil, India) were used as glass surfaces.

Biofilm Formation On Medical Devices:

Sterile latex catheters (CT) and enteral feeding tubes (EFT) were cut into 0.5 mm thick discs and were aseptically introduced into 5 ml YESCA broth inoculated with *E. cloacae* SBP-8 and the mutant strain (diluted to 1:100). The tubes were incubated for different periods (24, 48, 72 and 96 hrs) under static conditions. After respective incubation periods, unattached cells were removed by rinsing the discs of the enteral feeding tube and latex catheter with PBS. The discs were further stained with 1 ml of 0.1% crystal violet. After 30 min of incubation, the crystal violet solution was removed, and the excess stain was rinsed off with a mild wash by PBS buffer. Finally, the biofilm was extracted with 1 ml of 33% glacial acetic acid. After 30 min of incubation, the absorbance of the extracted solution was measured at 570 nm. The resulting absorbance is an indication of the of formed biofilm (Philips et al., 2017). The extracted solution was diluted 2 to 3 times before measurement. The optical densities were measured using Thermo scientific, multiscan Go spectrophotometer. All biofilm quantification experiments were done in triplicate.

Biofilm Formation On Glass Surface:

Glass tubes (1 cm diameter) were used to evaluate the biofilm formation. The tube containing 5 ml YESCA broth was inoculated with the 1% volume of the overnight grown culture of *E. cloacae* SBP-8 and the mutant strain. The tubes were incubated at 25°C and 37°C for different time intervals, as described

above, under static conditions. The crystal violet assays to evaluate biofilm formation was performed as described above.

Extraction Of Rna From Biofilm:

For RNA isolation, strains were grown in YESCA media in a static condition at 25°C and 37°C. RNA was isolated from biofilm formed as a ring in the sides of culture tubes using the conventional method (Ares, 2012) with minor modifications. The rings were scrapped from 5 tubes using a sterile cell scraper. The pooled rings were immediately mixed with 70% of ammonium sulfate (Merck) and incubated at RT for 5 min, followed by centrifugation at 5000g for 5 min at 4°C (Eppendorf, Germany). The supernatant was poured off, and the cells were digested with freshly prepared lysozyme (0.5mg/ml) and incubated at RT for 15 min. After digestion of the cells, 15µl of sodium acetate (CDH, India) and 45 µl of 10% SDS (Sigma, USA) was added and vortexed briefly for 10 sec. After this, 400 µl of P:C:I (Phenol: Chloroform: Isopropanol) solution was added, vortexed to emulsify, and incubated for 10 min at 65°C. The tube was incubated on ice for 5 minutes and centrifuged at 14000 g for 20 minutes. The resulting aqueous phase was extracted with an equal volume of chloroform (SRL), and the process was repeated. Finally, the aqueous phase was collected in a fresh tube, and RNA was precipitated using 50µl of sodium acetate and 100% ethanol (Merck) at -80°C for overnight. After overnight incubation, the samples were centrifuged at 14000 g for 30 min. The pellet was washed twice with 70% of ethanol and resuspended in 40 µl of water. For both medical devices, firstly, the culture was removed, and the entire surface (0.5 cm piece) was washed with 1X PBS to remove the unbound cells as adapted from (Mandakhalikar et al., 2018). The RNA from the biofilm was extracted as above. RNA samples were checked by agarose gel electrophoresis to assess the lack of degradation and quantified spectrophotometrically. Reverse transcription was performed with 800 ng of total RNA using Verso cDNA Synthesis Kit (Catalog number: AB/1453-A) as per the instruction from the manufacturer. cDNA synthesis efficiency was verified by electrophoresis on agarose gel using gene specific primer for curli.

Gene Expression Analysis:

The curli, functional amyloid, are known to play a pivotal role in initial adhesion and attachment. Hence, we were interested in exploring the involvement of curli on three abiotic surfaces (foley latex catheter, enteral feeding tube, and glass surface). We investigated the expression of *csgA*, one of the curli proteins, in *E. cloacae* SBP-8 at two different temperatures. Quantitative real-time PCR (qPCR) was performed using Bio-Rad SYBR green dye in Bio-Rad thermocycler using gene-specific primer for curli. Relative fold change in target gene expression was calculated using $2^{-\Delta\Delta}$ Ct method by normalizing against 16S rRNA. The expression of *csgA* was determined by using the delta Ct method (2ΔCt), a variation of the Livak method, where ΔCt = Ct (reference gene)-Ct (target gene) (França et al., 2011). All reactions were performed in duplicates, including negative control samples, which never showed significant threshold cycles. The primers used in the study are mentioned in the (**Supplementary material: Table 1).**

Confocal Laser Scanning Microscopy (Clsm) Of Biofilm:

Intact biofilms of *E. cloacae* were visualized using an inverted Zeiss (LSM) 880 Airyscan (Carl Zeiss, Jena, Germany) equipped with a C-APOCHROMAT 40x/1.2 Water Corr-UVVIS-IR objective. After t incubation for 48 to 72 hrs, the glass slides were removed from the culture tube and washed twice with 1X PBS in order to remove the unbound cells. To visualize the formed biofilm, the wild-type and mutant strain were stained with 5 µM SYTO9 (Thermoscientific) at room temperature for 30 min, followed by two 10-min washes with 1X PBS. The laser was used at 488 nm for excitation, and the emission was observed at 528 nm (SYTO9). Obtained images and z-stack projections were visualized using Zeiss ZEN System imaging software (Zeiss).

Observation Of Biofilm By Field Emission Scanning Electron Microscopy (Fesem):

We were further interested in visualizing the morphology of biofilm formed on the surfaces of latex catheters, glass slides, and the enteral feeding tube employing FESEM using the protocol of (Djeribi et al., 2012) with a minor modification. 0.5 mm thick discs of the medical devices and glass slides were aseptically introduced into tubes containing LB broth inoculated with *E. cloacae* SBP-*8* with a dilution of (1:100) at both 25°and 37°C under static conditions. The samples were dehydrated and sputter-coated with chromium metal using the Quorum Q150T ES system, and the morphology of the biofilm was observed in a Thermo scientific FEI FE-SEM APREO S SEM (Netherland) system at 20k V.

Statistical analysis:

All experiments were carried out in triplicates and repeated in three independent trial sets. Statistical analysis was performed using Prism 7 (Graph Pad Software). Unpaired Student's t-test was performed. Statistical significance: *, $P \leq 0.05$, **, $P \leq 0.01$, ***, $P \leq 0.001$, ****, $P < 0.0001$, ns = not significant

Results

Phenotypic characterization of curli:

To understand the role of curli in biofilm formation, we generated a *csgA* mutant. Firstly we used a standard qualitative method to distinguish curli-producing bacteria from non-curliated bacteria, where CR-binding is known to be curli-dependent (Reichhardt et al., 2015). The wild-type strain produced (left panel) deep red colouration in comparison to the knockout strain (right panel), which did not develop red phenotype on Congo red media at any incubation temperature used in the study, indicating the absence of curli (Fig. 1A). We also tested for the curli production at 37°C which is represented in (Fig. 1B) which showed light red (left panel) and light pink-white colour (right panel) by wild type and the mutant respectively. In the wild-type strain, the curli production was observed at 25°C for 24–96 hrs. Although we

found lesser colouration for 37°C on the CR-YESCA plate, we further examined the role of curli at the temperature in reference to the biofilm formation at varying surfaces.

Contribution of curli to E. cloacae **SBP-8 biofilm formation on abiotic surfaces**:

We first evaluated the biofilm formation of *E. cloacae* SBP-8 wild-type strain and *csgA* mutant on different abiotic surfaces where cells were grown in YESCA broth at 25°C, a condition known to induce the biogenesis of curli. It was also tested at 37°C to test if the curli are produced at this temperature and potentially contribute to the biofilm formation on abiotic surfaces. To ascertain that *csgA* deletion does not affect growth of the bacteria, growth kinetics was performed, which did not show a significant growth difference in wild-type and Δ*csg* knockout (**Fig S1)**.

We used a crystal violet (CV) assay to evaluate the biofilm formed on various surfaces, which is the most suitable method to quantify the biofilms (Philips et al., 2017). The initial examination of the crystal violet tubes at both 25 and 37°C revealed a difference in the adhered biomass (ring), which indicated that the wild-type strain adhered more in comparison to the mutant strain mentioned in the supplementary material (**Fig S2)**. Generally, the biofilm formation on the glass surface was less intense than the other two surfaces at 25°C (Fig. 2A-C**)**. Deletion of *csgA* negatively affected the biofilm formation on all three surfaces used. However, the percentage decrease upon *csgA* deletion observed was different at different time intervals and types of surfaces. CV staining showed 48 and 21.5% decrease in biofilm formation by Δ*csgA* strain on the glass surface at 72 and 48 hrs, respectively. For the enteral feeding tube, we found significant differences at only 48 hrs (Fig. 2B**).** In contrast, for the foley latex catheter, we found significant differences from 48–96 hrs, as clearly shown in Fig. 2C. In the case of the medical devices, we found 49% reduction in the biofilm at 48 hrs on an enteral feeding tube (Fig. 2B). The latex catheter showed 29 and 58% reduction respectively at 48 and 74 hrs in biofilm formed by Δ*csgA* strain as compared to its wild-type counterpart (Fig. 2C). Similar results were obtained at 37°C where deletion of *csgA* caused decreased biofilm formation by *E. cloacae* SBP-8 (Fig. 2D-F**)**. However, the effect of *csgA* deletion on biofilm formation was less severe at 37°C than at 25°C. The Δ*csgA* strain showed 23–27%, 11–21%, and 13 to 29% decrease in biofilm on glass, enteral feeding tube, and latex catheter, respectively, at different time intervals.

Morphological Analysis Of The Biofilms Through Scanning Electron Microscopy:

To visualize the fine architecture and cell aggregation of the formed biofilms on glass surface and medical devices (enteral feeding tube and latex Foley catheter), FESEM analysis was performed. As seen in Fig. 3, 4, and 5, the surfaces were readily and rapidly colonized by a wild-type strain of *E. cloacae* SBP-8. In comparison to the glass surface, the medical devices formed multi-layers of cells encased in dense uniform EPS, indicating more biofilm formation on medical devices rather than the glass surface. On the contrary, the mutant strain (*ΔcsgA*) exhibited lesser colonization with sparsely distributed cells on the surface **(**Fig. 3, 4, and 5**).** The mutant strains exhibited diffuse monolayers at all intervals, along with

lesser EPS production. Moreover, we observed that the mutant strains showed elongated cells and flat morphology (Azam et al., 2020). Thus, our result indicated that curli is critical for the firm attachment of cells onto the surface and for the three-dimensional development of biofilms in *E. cloacae* SBP-8

Further, we performed confocal scanning laser microscopy analysis on the biofilms grown on glass slides at 25°C and 37°C to validate the architecture. The biofilms were grown on glass slides. Both wild-type and mutant strains were incubated at static 25°C and 37°C temperatures. The samples were stained with Syto-9 (nucleic acid binding dye) and analysed after the incubation period of 48 to 72 hrs. It was seen that wild type strain had a dense population of cells that covered the entire glass surface at both the temperature, as shown in Fig. 6. At 48 hrs **(A)** and 72 hrs **(B)**, the wild-type strain exhibited strong colonization to the surface and clearly showed micro-colony formation with strong biofilms formed at other regions, which is clear representation of the phase switch from micro-colonies to sessile state. On the contrary, the mutant strain showed weaker adherence and colonization to the surface with diffused monolayers of cells with the least EPS production at both temperatures (Fig. 6)

Curli Plays A Role In Initial Attachment To Abiotic Surfaces:

We tested the role of curli in the biofilm formation at both temperatures 25°and 37°C through real-time qRT-PCR. We found the curli expression at both temperatures on all the surfaces. For 25°C we found curli expression to be significantly increasing from 72 to 96 hrs ($p > 0.0442$) (Fig. 7A**)**. In the enteral feeding tube, we found a significant increase from 48 to72 hrs ($p > 0.0442$) (Fig. 7B**)**. For foley latex catheter we found the curli expression to be increasing at 72 to 96 hrs ($p > 0.0442$) (Fig. 7C**)**. The gene expression studies were also carried out at 37°C. For the glass surface (Fig. 7D**)**, we found a significant increase of *csgA* gene expression from 48 to 72 hrs ($p > 0.0048$) followed by a decrease from 72 to 96 hrs ($p > 0.0048$). For the enteral feeding tube (Fig. 7E**)**, there was higher expression of *csgA* at 48 hrs which significantly ($p > 0.0048$) decreased at 72 to 96 hrs ($p > 0.0442$). Similarly, for the foley latex catheter (Fig. 7F**)**, there was a significant increase from 48 to 72 hrs

Discussion

E. cloacae, a member of the ESKAPE group, is one of the important nosocomial pathogens which can form biofilms on abiotic surfaces, including medical devices. Biofilm formation is a highly regulated, complex, and dynamic process mediated by multiple genetic, physiological, and environmental factors. Since biofilm formation is one of the key entities which imparts virulence to most of the organisms, the present study investigated the potential role of curli, an important proteinaceous component of the biofilm matrix, in the biofilm formation of an pathogenic environmental isolate (non-clinical) of *E. cloacae* (SBP-8) (Khan et al., 2020). To the best of our knowledge, the role of curli in biofilm formation by *E. cloacae* is underexplored. To understand the role of curli, we generated a knockout mutant of *csgA*, the structural protein, and monomer of curli fimbriae, employing red-recombinase system.

The preliminary screening of curli using CR assay confirmed the curli production by *E. cloacae* SBP-8 at both 25 and 37°C. Since CR can also bind to other bacterial factors like cellulose, we used Δ*csgA* mutant of *E. cloacae.* The loss of colour in the mutant bacterial colony confirmed that the colour change on CR-agar plate was due to curli production (Fig. 1). CR assay is a standard method for curli production and has been used to confirm it in *E. coli* (Reichhardt et al., 2015). It has also been well-documented in *E.coli* O157:H7 that congo red affinity is strongly dependent on the curli production at both 25 and 37°C temperatures (Sharma and Bearson, 2013). Similar findings have been reported in various members from the family of *Enterobacteriaceae* which includes to enterotoxigenic *E. coli, Citrobacter freundii, C. koseri/farmeri, E. aerogenes, E. cloacae, E. sakazakii, Klebsiella oxytoca, K. pneumoniae, Proteus mirabilis*, and *Raoultella ornithinolytica* which showed binding to Congo red 25 and 37°C (Zogaj et al., 2003; Szabó et al., 2005).

To investigate the role of curli in biofilm production by *E. cloacae* SBP-8, crystal violet (CV) assay was performed under static conditions at 25 and 37°C. Decreased biofilm formation in Δ*csgA* mutant at both temperatures and all the surfaces clearly indicated that curli does play a role in biofilm formation by *E. cloacae.* A similar observation was reported in *E. coli* K-12 and *E. coli* strain MG1655, where curli deficient strains adhered less to the surface (Beloin et al., 2008; Azam et al., 2020). Another report on *E. coli* also showed that the curli facilitates attachment and provides a scaffold for the maturation of biofilms, where it was shown to adhere to polyurethane sheets at both 25 and 37°C (16). However, the effect of *csg* gene deletion on biofilm formation was highest between 48 to 72 hrs of biofilm development at different surfaces. It highlights that curli is primarily required for adhesion during the initiation of biofilm formation and the development of multi-layered cell clusters on various surfaces (Prigent-Combaret et al., 2000; Le Thi et al., 2001). Similar studies have shown that curli is important for different strains of *E. coli*, including O157:H7 and STEC (Shiga toxin-producing *E. coli*) to attach to the biotic and abiotic surfaces such as glass, stainless steel, polystyrene, and stainless steel (Cookson et al., 2002; Jain and Chen, 2007; Uhlich et al., 2009). Another study demonstrated that attachment and biofilm formation on glass and polystyrene surfaces by curli-producing *Salmonella* strains was more efficient than non-curli-producing strains (Austin et al., 1998).

To correlate the role of curli with biofilm formation at the molecular level, we further quantified the expression of *csgA*, a structural gene of curli at 25 and 37°C. In general, the curli fibers are known to be expressed at a lower temperature (25–30°C) as observed in our study as well, but not at 37°C in various enteric bacteria (Kikuchi et al., 2005). On the contrary, we observed *csgA* expression also at 37°C on all the surfaces mentioned above. Our observation aligned with the study carried out in different pathogenic strains of *E. coli* only including six pathogenic isolates of *E. coli* O:157, *E. coli* O157:H7 (Ben Nasr et al., 1996; Uhlich et al., 2001; Gualdi et al., 2008). Similar findings have been reported in STEC *E. coli*, where it has been demonstrated that the maturation of the biofilm cannot take place without the expression of curli at 37°C (Dewanti and Wong, 1995). As mentioned earlier, curli is expressed at 37°C in the case of *E. coli* K-12, helping in a strong attachment to the polyurethane sheets (Kikuchi et al., 2005). Our results infer that expression of the curli at 37°C is favourable for the organism to adhere to the biotic and abiotic surfaces and is thought to be strain-dependent owing to the complex regulatory network governing curli

biogenesis (Gophna et al., 2001). Natural variation in curli production has been reported for *E. coli* isolates and STEC O157:H7 populations originating from diverse environments (Maurer et al., 1998; Scheuerman et al., 1998; Carter et al., 2016).

Our gene expression analysis also showed that curli is required for attachment on the above-mentioned surfaces at both temperatures. We found increased curli expression on the glass surface, an enteral feeding tube (PVC), and a latex foley catheter. Medical implants, including catheters and stents implanted into the human body, are primarily vulnerable to colonization by biofilms (Austin et al., 1998). Various findings have shown that curli helps in attachment to hydrophilic surfaces. Biomaterials owing to their roughness and irregularities in the polymeric substances, provide easy bacterial adhesion and attachment to the bacterial colonies. Our data also significantly depicted that biofilm formation was more on medical devices than the glass surface (Eginton et al., 1995; Scheuerman et al., 1998). It has been reported that particular surface structures, such as type I secretion system and curli fimbriae, enable the cells to stabilize onto the surface (Ryu et al., 2004).

The overall reduction in the biofilm formation by *E. cloacae* SBP-8 Δ*csgA* gives us an insight that the curli in enteric bacteria could potentially contribute towards the colonization on various surfaces. Although not yet formally demonstrated, our observations support the notion that curli could indeed be expressed by *E. cloacae* SBP-8 growing *in-vivo*, especially in biofilm, where environmental conditions and selective pressures are considerably different from *in vitro* conditions. Thus, once expressed, curli will have the capacity to interact with human cells at physiological temperatures and contribute to the pathophysiology of bacterial infectious disease.

Our CLSM analysis of biofilm formed by *E. coli* strain W3110 under static conditions at 30°C revealed that the curli is restricted to the micro-colony formation stages. In contrast, the mutant Δ*csgA* strain showed lesser adhesion to the surface with low cell density (Besharova et al., 2016).

Curli have been identified as a morphological envelope structure of major importance for biofilm formation in *E.coli* (Vidal et al., 1998). Our findings through FESEM analysis demonstrated that curli is required for biofilm formation on various surfaces. Since the wild-type strain produced substantially thicker layers of cells than the mutant strains, it appears that curli stabilizes intracellular contacts allowing aggregation to occur and thereby increasing the biofilm thickness (Austin et al., 1998). Similar to the function of thin aggregative fimbriae, curli produced by diarrheagenic *E. coli* plays an influential role by forming stiff colony phenotypes and cell clumping to facilitate strong adhesion (Collinson et al., 1992). Similar to our observations, several studies using SEM and TEM have shown a clear role of curli in mediating cell-to-cell contact and forming thick bundles which bind cells together onto the surface in enteropathogenic *E. coli* (Giron et al., 1991). Auto aggregation was shown to be high in *ompR234 E. coli* K-12, suggesting that this property results from the formation of interbacterial curli bundles formation (Sohel et al., 1996; Vidal et al., 1998).

Altogether, the results obtained in this study allow us to draw up a model that explains biofilm development on abiotic surfaces. Curli plays a fundamental role in two steps required for biofilm

development: initial bacterial attachment and three-dimensional biofilm formation. Curli helps in adhesion and becomes an important requirement by the bacterial cells to attach to the varying surface with different physio-chemical properties. Although curli have been shown to mediate the binding of host proteins in the mammalian host, we speculate that the varying conditions encountered outside the host enhance curli expression. Our study shows that curli are more precisely adapted to bacterial attachment on inert surfaces. To cope successfully with diverse environmental conditions, it seems convincing that *E. cloacae* SBP-8 may possess adaptive programs for optimizing growth and survival in each ecological niche as demonstrated through our study.

Conclusions

The present work demonstrates the production of curli and its role in biofilm formation at various abiotic surfaces, including medical devices by *E. cloacae* SBP-8, an environmental isolate. Despite being from the exogenous origin (not from a clinical setup), the isolate forms a strong biofilm on various surfaces indicating the potential of environmental *E. cloacae* to en route immunocompromised patients through medical device-associated microbial contaminations. *E. cloacae* SBP-8 forms biofilm on the tested medical devices and glass surfaces with varied intensity. With the deletion of *csgA* gene encoding curli, the biofilm formation is significantly affected, particularly at the initial time (24–72 hrs), which suggests that the curli is required for initiation of biofilm formation during the adherence of microbial cells on the surfaces. Our results suggest that curli production is important in the process of initial attachment and conferring cell aggregation property to *E. cloacae* SBP-8 on abiotic materials. It can be speculated that the functional amyloid of *E. cloacae* SBP-8 is one of the key factors required for to successful colonization on different materials and thereby contributes to the Hospital Associated Infection through contaminated medical devices. Hence curli fibres serves as a potential target for biofilm interventions and infection control.

Declarations

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Author's contribution:

Conceptualization: TM and PNJ; methodology: TM ; software: TM and MT ; validation: TM and PNJ; formal analysis: TM, PNJ ; investigation: TM and PNJ; resources: PNJ .; data curation, TM and PNJ.; writing—original draft preparation, TM and MT ; writing—review and editing, TM, PNJ, MT.; visualization, PNJ, MT.; supervision, PNJ MT.; project administration, PNJ.;. All authors have read and agreed to the published version of the manuscript.

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References

1. Ares, M. 2012. Bacterial RNA isolation. *Cold spring harbor protocols* 2012, pdb. prot071068.
2. Austin, J.W., Sanders, G., Kay, W.W., and Collinson, S.K. 1998. Thin aggregative fimbriae enhance Salmonella enteritidis biofilm formation. FEMS microbiology letters 162, 295–301.
3. Azam, M.W., Zuberi, A., and Khan, A.U. 2020. bolA gene involved in curli amyloids and fimbriae production in E. coli: exploring pathways to inhibit biofilm and amyloid formation. Journal of Biological Research-Thessaloniki 27, 1–12.
4. Barnhart, M.M., and Chapman, M.R. 2006. Curli biogenesis and function. Annu. Rev. Microbiol. 60, 131–147.
5. Beloin, C., Houry, A., Froment, M., Ghigo, J.-M., and Henry, N. 2008. A short–time scale colloidal system reveals early bacterial adhesion dynamics. PLoS biology 6, e167.
6. Ben Nasr, A., Olsén, A., Sjöbring, U., Müller-Esterl, W., and Björck, L. 1996. Assembly of human contact phase proteins and release of bradykinin at the surface of curli-expressing Escherichia coli. Molecular microbiology 20, 927–935.
7. Besharova, O., Suchanek, V.M., Hartmann, R., Drescher, K., and Sourjik, V. 2016. Diversification of gene expression during formation of static submerged biofilms by Escherichia coli. Frontiers in microbiology 7, 1568.
8. Carter, M.Q., Louie, J.W., Feng, D., Zhong, W., and Brandl, M.T. 2016. Curli fimbriae are conditionally required in Escherichia coli O157: H7 for initial attachment and biofilm formation. Food microbiology 57, 81–89.
9. Collinson, S., Emödy, L., Trust, T., and Kay, W. 1992. Thin aggregative fimbriae from diarrheagenic Escherichia coli. Journal of bacteriology 174, 4490–4495.

10. Cookson, A.L., Cooley, W.A., and Woodward, M.J. 2002. The role of type 1 and curli fimbriae of Shiga toxin-producing Escherichia coli in adherence to abiotic surfaces. International Journal of Medical Microbiology 292, 195–205.
11. Costerton, J.W., Cheng, K., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., and Marrie, T.J. 1987. Bacterial biofilms in nature and disease. Annual Reviews in Microbiology 41, 435–464.
12. Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M. 1995. Microbial biofilms. Annual review of microbiology 49, 711–745.
13. Davies, D. 2003. Understanding biofilm resistance to antibacterial agents. Nature reviews Drug discovery 2, 114–122.
14. Dewanti, R., and Wong, A.C. 1995. Influence of culture conditions on biofilm formation by Escherichia coli O157: H7. International journal of food microbiology 26, 147–164.
15. Djeribi, R., Bouchloukh, W., Jouenne, T., and Menaa, B. 2012. Characterization of bacterial biofilms formed on urinary catheters. American journal of infection control 40, 854–859.
16. Eginton, P., Gibson, H., Holah, J., Handley, P., and Gilbert, P. 1995. The influence of substratum properties on the attachment of bacterial cells. Colloids and Surfaces B: Biointerfaces 5, 153–159.
17. Evans, M.L., and Chapman, M.R. 2014. Curli biogenesis: order out of disorder. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1843, 1551–1558.
18. Flemming, H.-C., and Wingender, J. 2010. The biofilm matrix. Nature reviews microbiology 8, 623–633.
19. França, A., Melo, L.D., and Cerca, N. 2011. Comparison of RNA extraction methods from biofilm samples of Staphylococcus epidermidis. BMC research notes 4, 1–5.
20. Giron, J.A., Ho, A., and Schoolnik, G.K. 1991. An inducible bundle-forming pilus of enteropathogenic Escherichia coli. Science 254, 710–713.
21. Gophna, U., Barlev, M., Seijffers, R., Oelschlager, T., Hacker, J., and Ron, E. 2001. Curli fibers mediate internalization of Escherichia coli by eukaryotic cells. Infection and immunity 69, 2659–2665.
22. Goulter, R.M., Gentle, I.R., and Dykes, G.A. 2010. Characterisation of curli production, cell surface hydrophobicity, autoaggregation and attachment behaviour of Escherichia coli O157. Current microbiology 61, 157–162.
23. Gualdi, L., Tagliabue, L., Bertagnoli, S., Ierano, T., De Castro, C., and Landini, P. 2008. Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in Escherichia coli. Microbiology 154, 2017–2024.
24. Huang, T.-W., Lam, I., Chang, H.-Y., Tsai, S.-F., Palsson, B.O., and Charusanti, P. 2014. Capsule deletion via a λ-Red knockout system perturbs biofilm formation and fimbriae expression in Klebsiella pneumoniae MGH 78578. BMC research notes 7, 1–8.
25. Jain, S., and Chen, J. 2007. Attachment and biofilm formation by various serotypes of Salmonella as influenced by cellulose production and thin aggregative fimbriae biosynthesis. Journal of food protection 70, 2473–2479.

26. Khan, S., Paravastu, P., Jha, P.N., and Marathe, S.A. 2020. Elucidating the pathogenic potential of Enterobacter cloacae SBP-8 using Caenorhabditis elegans as a model host. Microbial Pathogenesis 148, 104449.
27. Kikuchi, T., Mizunoe, Y., Takade, A., Naito, S., and Yoshida, S.i. 2005. Curli fibers are required for development of biofilm architecture in Escherichia coli K-12 and enhance bacterial adherence to human uroepithelial cells. Microbiology and immunology 49, 875–884.
28. Le Thi, T.-T., Prigent-Combaret, C., Dorel, C., and Lejeune, P. (2001) [15] First stages of biofilm formation: Characterization and quantification of bacterial functions involved in colonization process. In *Methods in Enzymology*. Elsevier, Vol. 336, pp. 152–159.
29. Mandakhalikar, K.D., Rahmat, J.N., Chiong, E., Neoh, K.G., Shen, L., and Tambyah, P.A. 2018. Extraction and quantification of biofilm bacteria: Method optimized for urinary catheters. Scientific reports 8, 1–9.
30. Maurer, J.J., Brown, T.P., Steffens, W., and Thayer, S.G. 1998. The occurrence of ambient temperature-regulated adhesins, curli, and the temperature-sensitive hemagglutinin tsh among avian Escherichia coli. Avian diseases, 106–118.
31. McWilliams, B., and Torres, A. EHEC Adhesins. Microbiol. Spectr. 2014; 2: EHEC00032013. doi: 10.1128/microbiolspec. EHEC-0003-2013.[PMC free article][PubMed][CrossRef][Google Scholar].
32. Nyenje, M.E., Green, E., and Ndip, R.N. 2013. Evaluation of the effect of different growth media and temperature on the suitability of biofilm formation by Enterobacter cloacae strains isolated from food samples in South Africa. Molecules 18, 9582–9593.
33. Philips, J., Rabaey, K., Lovley, D.R., and Vargas, M. 2017. Biofilm formation by Clostridium ljungdahlii is induced by sodium chloride stress: experimental evaluation and transcriptome analysis. PLoS One 12, e0170406.
34. Piozzi, A., Francolini, I., Occhiaperti, L., Di Rosa, R., Ruggeri, V., and Donelli, G. 2004. Polyurethanes loaded with antibiotics: influence of polymer-antibiotic interactions on in vitro activity against Staphylococcus epidermidis. Journal of chemotherapy 16, 446–452.
35. Prigent-Combaret, C., Prensier, G., Le Thi, T.T., Vidal, O., Lejeune, P., and Dorel, C. 2000. Developmental pathway for biofilm formation in curli-producing Escherichia coli strains: role of flagella, curli and colanic acid. Environmental microbiology 2, 450–464.
36. Reichhardt, C., Jacobson, A.N., Maher, M.C., Uang, J., McCrate, O.A., Eckart, M., and Cegelski, L. 2015. Congo red interactions with curli-producing E. coli and native curli amyloid fibers. PloS one 10, e0140388.
37. Roy, R., Tiwari, M., Donelli, G., and Tiwari, V. 2018. Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. Virulence 9, 522–554.
38. Ryu, J.H., Kim, H., Frank, J., and Beuchat, L. 2004. Attachment and biofilm formation on stainless steel by Escherichia coli O157: H7 as affected by curli production. Letters in applied microbiology 39, 359–362.

39. Santajit, S., and Indrawattana, N. 2016. Mechanisms of antimicrobial resistance in ESKAPE pathogens. *BioMed research international* 2016.
40. Sawitzke, J.A., Thomason, L.C., Bubunenko, M., Li, X., Costantino, N., and Court, D.L. (2013) Recombineering: using drug cassettes to knock out genes in vivo. In *Methods in enzymology*. Elsevier, Vol. 533, pp. 79–102.
41. Scheuerman, T.R., Camper, A.K., **and** Hamilton, M.A. 1998. Effects of substratum topography on bacterial adhesion. Journal of colloid and interface science **208**, 23–33.
42. Sharma, V.K., **and** Bearson, B.L. 2013. Hha controls Escherichia coli O157: H7 biofilm formation by differential regulation of global transcriptional regulators FlhDC and CsgD. Applied and environmental microbiology **79**, 2384–2396.
43. Singh, P., Schaefer, A., Parsek, M., Moninger, T., Welsh, M., and Greenberg, E. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature407: 762–764.
44. Singh, R.P., Nalwaya, S., **and** Jha, P.N. 2017. The draft genome sequence of the plant growth promoting rhizospheric bacterium Enterobacter cloacae SBP-8. Genomics data **12**, 81–83.
45. Sohel, I., Puente, J.L., Ramer, S.W., Bieber, D., Wu, C.-Y., **and** Schoolnik, G.K. 1996. Enteropathogenic Escherichia coli: identification of a gene cluster coding for bundle-forming pilus morphogenesis. Journal of bacteriology **178**, 2613–2628.
46. Szabó, E., Skedsmo, A., Sonnevend, A., Al-Dhaheri, K., Emödy, L., Usmani, A., **and** Pal, T. 2005. Curli expression of enterotoxigenicEscherichia coli. Folia microbiologica **50**, 40–46.
47. Uhlich, G.A., Gunther IV, N.W., Bayles, D.O., **and** Mosier, D.A. 2009. The CsgA and Lpp proteins of an Escherichia coli O157: H7 strain affect HEp-2 cell invasion, motility, and biofilm formation. Infection and immunity **77**, 1543–1552.
48. Uhlich, G.A., Keen, J.E., **and** Elder, R.O. 2001. Mutations in the csgD promoter associated with variations in curli expression in certain strains of Escherichia coli O157: H7. Applied and environmental microbiology **67**, 2367–2370.
49. **Van** Gerven, N., **Van der** Verren, S.E., Reiter, D.M., **and** Remaut, H. 2018. The role of functional amyloids in bacterial virulence. Journal of molecular biology **430**, 3657–3684.
50. Vidal, O., Longin, R., Prigent-Combaret, C., Dorel, C., Hooreman, M., **and** Lejeune, P. 1998. Isolation of an Escherichia coli K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new ompR allele that increases curli expression. Journal of bacteriology **180**, 2442–2449.
51. Wu, H., Moser, C., Wang, H.-Z., Høiby, N., **and** Song, Z.-**J**. 2015. Strategies for combating bacterial biofilm infections. *International journal of oral science* **7**, 1–7.
52. Zhang, D., He, W., Tong, Q., Zhou, J., **and** Su, X. 2016. Multi-omics analysis on the pathogenicity of Enterobacter cloacae ENHKU01 isolated from sewage outfalls along the Ningbo coastline. Proteome science **14**, 1–14.
53. Zhou, Y., Smith, D.R., Hufnagel, D.A., and Chapman, M.R. (2013) Experimental manipulation of the microbial functional amyloid called curli. In *Bacterial cell surfaces*. Springer, pp. 53–75.

54. Zogaj, X., Bokranz, W., Nimtz, M., **and** Römling, U. 2003. Production of cellulose and curli fimbriae by members of the family Enterobacteriaceae isolated from the human gastrointestinal tract. Infection and immunity **71**, 4151–4158.
55. Ares, M. 2012. Bacterial RNA isolation. *Cold spring harbor protocols* 2012, pdb. prot071068.
56. Austin, J.W., Sanders, G., Kay, W.W., and Collinson, S.K. 1998. Thin aggregative fimbriae enhance Salmonella enteritidis biofilm formation. FEMS microbiology letters 162, 295–301.
57. Azam, M.W., Zuberi, A., and Khan, A.U. 2020. bolA gene involved in curli amyloids and fimbriae production in E. coli: exploring pathways to inhibit biofilm and amyloid formation. Journal of Biological Research-Thessaloniki 27, 1–12.
58. Barnhart, M.M., and Chapman, M.R. 2006. Curli biogenesis and function. Annu. Rev. Microbiol. 60, 131–147.
59. Beloin, C., Houry, A., Froment, M., Ghigo, J.-M., and Henry, N. 2008. A short–time scale colloidal system reveals early bacterial adhesion dynamics. PLoS biology 6, e167.
60. Ben Nasr, A., Olsén, A., Sjöbring, U., Müller-Esterl, W., and Björck, L. 1996. Assembly of human contact phase proteins and release of bradykinin at the surface of curli-expressing Escherichia coli. Molecular microbiology 20, 927–935.
61. Besharova, O., Suchanek, V.M., Hartmann, R., Drescher, K., and Sourjik, V. 2016. Diversification of gene expression during formation of static submerged biofilms by Escherichia coli. Frontiers in microbiology 7, 1568.
62. Carter, M.Q., Louie, J.W., Feng, D., Zhong, W., and Brandl, M.T. 2016. Curli fimbriae are conditionally required in Escherichia coli O157: H7 for initial attachment and biofilm formation. Food microbiology 57, 81–89.
63. Collinson, S., Emödy, L., Trust, T., and Kay, W. 1992. Thin aggregative fimbriae from diarrheagenic Escherichia coli. Journal of bacteriology 174, 4490–4495.
64. Cookson, A.L., Cooley, W.A., and Woodward, M.J. 2002. The role of type 1 and curli fimbriae of Shiga toxin-producing Escherichia coli in adherence to abiotic surfaces. International Journal of Medical Microbiology 292, 195–205.
65. Costerton, J.W., Cheng, K., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., and Marrie, T.J. 1987. Bacterial biofilms in nature and disease. Annual Reviews in Microbiology 41, 435–464.
66. Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M. 1995. Microbial biofilms. Annual review of microbiology 49, 711–745.
67. Davies, D. 2003. Understanding biofilm resistance to antibacterial agents. Nature reviews Drug discovery 2, 114–122.
68. Dewanti, R., and Wong, A.C. 1995. Influence of culture conditions on biofilm formation by Escherichia coli O157: H7. International journal of food microbiology 26, 147–164.
69. Djeribi, R., Bouchloukh, W., Jouenne, T., and Menaa, B. 2012. Characterization of bacterial biofilms formed on urinary catheters. American journal of infection control 40, 854–859.

70. Eginton, P., Gibson, H., Holah, J., Handley, P., and Gilbert, P. 1995. The influence of substratum properties on the attachment of bacterial cells. Colloids and Surfaces B: Biointerfaces 5, 153–159.
71. Evans, M.L., and Chapman, M.R. 2014. Curli biogenesis: order out of disorder. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1843, 1551–1558.
72. Flemming, H.-C., and Wingender, J. 2010. The biofilm matrix. Nature reviews microbiology 8, 623–633.
73. França, A., Melo, L.D., and Cerca, N. 2011. Comparison of RNA extraction methods from biofilm samples of Staphylococcus epidermidis. BMC research notes 4, 1–5.
74. Giron, J.A., Ho, A., and Schoolnik, G.K. 1991. An inducible bundle-forming pilus of enteropathogenic Escherichia coli. Science 254, 710–713.
75. Gophna, U., Barlev, M., Seijffers, R., Oelschlager, T., Hacker, J., and Ron, E. 2001. Curli fibers mediate internalization of Escherichia coli by eukaryotic cells. Infection and immunity 69, 2659–2665.
76. Goulter, R.M., Gentle, I.R., and Dykes, G.A. 2010. Characterisation of curli production, cell surface hydrophobicity, autoaggregation and attachment behaviour of Escherichia coli O157. Current microbiology 61, 157–162.
77. Gualdi, L., Tagliabue, L., Bertagnoli, S., Ierano, T., De Castro, C., and Landini, P. 2008. Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in Escherichia coli. Microbiology 154, 2017–2024.
78. Huang, T.-W., Lam, I., Chang, H.-Y., Tsai, S.-F., Palsson, B.O., and Charusanti, P. 2014. Capsule deletion via a λ-Red knockout system perturbs biofilm formation and fimbriae expression in Klebsiella pneumoniae MGH 78578. BMC research notes 7, 1–8.
79. Jain, S., and Chen, J. 2007. Attachment and biofilm formation by various serotypes of Salmonella as influenced by cellulose production and thin aggregative fimbriae biosynthesis. Journal of food protection 70, 2473–2479.
80. Khan, S., Paravastu, P., Jha, P.N., and Marathe, S.A. 2020. Elucidating the pathogenic potential of Enterobacter cloacae SBP-8 using Caenorhabditis elegans as a model host. Microbial Pathogenesis 148, 104449.
81. Kikuchi, T., Mizunoe, Y., Takade, A., Naito, S., and Yoshida, S.i. 2005. Curli fibers are required for development of biofilm architecture in Escherichia coli K-12 and enhance bacterial adherence to human uroepithelial cells. Microbiology and immunology 49, 875–884.
82. Le Thi, T.-T., Prigent-Combaret, C., Dorel, C., and Lejeune, P. (2001) [15] First stages of biofilm formation: Characterization and quantification of bacterial functions involved in colonization process. In *Methods in Enzymology*. Elsevier, Vol. 336, pp. 152–159.
83. Mandakhalikar, K.D., Rahmat, J.N., Chiong, E., Neoh, K.G., Shen, L., and Tambyah, P.A. 2018. Extraction and quantification of biofilm bacteria: Method optimized for urinary catheters. Scientific reports 8, 1–9.
84. Maurer, J.J., Brown, T.P., Steffens, W., and Thayer, S.G. 1998. The occurrence of ambient temperature-regulated adhesins, curli, and the temperature-sensitive hemagglutinin tsh among avian Escherichia

coli. Avian diseases, 106–118.

85. McWilliams, B., and Torres, A. EHEC Adhesins. Microbiol. Spectr. 2014; 2: EHEC00032013. doi: 10.1128/microbiolspec. EHEC-0003-2013.[PMC free article][PubMed][CrossRef][Google Scholar].
86. Nyenje, M.E., Green, E., and Ndip, R.N. 2013. Evaluation of the effect of different growth media and temperature on the suitability of biofilm formation by Enterobacter cloacae strains isolated from food samples in South Africa. Molecules 18, 9582–9593.
87. Philips, J., Rabaey, K., Lovley, D.R., and Vargas, M. 2017. Biofilm formation by Clostridium ljungdahlii is induced by sodium chloride stress: experimental evaluation and transcriptome analysis. PLoS One 12, e0170406.
88. Piozzi, A., Francolini, I., Occhiaperti, L., Di Rosa, R., Ruggeri, V., and Donelli, G. 2004. Polyurethanes loaded with antibiotics: influence of polymer-antibiotic interactions on in vitro activity against Staphylococcus epidermidis. Journal of chemotherapy 16, 446–452.
89. Prigent-Combaret, C., Prensier, G., Le Thi, T.T., Vidal, O., Lejeune, P., and Dorel, C. 2000. Developmental pathway for biofilm formation in curli-producing Escherichia coli strains: role of flagella, curli and colanic acid. Environmental microbiology 2, 450–464.
90. Reichhardt, C., Jacobson, A.N., Maher, M.C., Uang, J., McCrate, O.A., Eckart, M., and Cegelski, L. 2015. Congo red interactions with curli-producing E. coli and native curli amyloid fibers. PloS one 10, e0140388.
91. Roy, R., Tiwari, M., Donelli, G., and Tiwari, V. 2018. Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. Virulence 9, 522–554.
92. Ryu, J.H., Kim, H., Frank, J., and Beuchat, L. 2004. Attachment and biofilm formation on stainless steel by Escherichia coli O157: H7 as affected by curli production. Letters in applied microbiology 39, 359–362.
93. Santajit, S., and Indrawattana, N. 2016. Mechanisms of antimicrobial resistance in ESKAPE pathogens. *BioMed research international* 2016.
94. Sawitzke, J.A., Thomason, L.C., Bubunenko, M., Li, X., Costantino, N., and Court, D.L. (2013) Recombineering: using drug cassettes to knock out genes in vivo. In *Methods in enzymology*. Elsevier, Vol. 533, pp. 79–102.
95. Scheuerman, T.R., Camper, A.K., and Hamilton, M.A. 1998. Effects of substratum topography on bacterial adhesion. Journal of colloid and interface science 208, 23–33.
96. Sharma, V.K., and Bearson, B.L. 2013. Hha controls Escherichia coli O157: H7 biofilm formation by differential regulation of global transcriptional regulators FlhDC and CsgD. Applied and environmental microbiology 79, 2384–2396.
97. Singh, P., Schaefer, A., Parsek, M., Moninger, T., Welsh, M., and Greenberg, E. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature407: 762–764.
98. Singh, R.P., Nalwaya, S., and Jha, P.N. 2017. The draft genome sequence of the plant growth promoting rhizospheric bacterium Enterobacter cloacae SBP-8. Genomics data 12, 81–83.

99. Sohel, I., Puente, J.L., Ramer, S.W., Bieber, D., Wu, C.-Y., and Schoolnik, G.K. 1996. Enteropathogenic Escherichia coli: identification of a gene cluster coding for bundle-forming pilus morphogenesis. Journal of bacteriology 178, 2613–2628.
100. Szabó, E., Skedsmo, A., Sonnevend, A., Al-Dhaheri, K., Emödy, L., Usmani, A., and Pal, T. 2005. Curli expression of enterotoxigenicEscherichia coli. Folia microbiologica 50, 40–46.
101. Uhlich, G.A., Gunther IV, N.W., Bayles, D.O., and Mosier, D.A. 2009. The CsgA and Lpp proteins of an Escherichia coli O157: H7 strain affect HEp-2 cell invasion, motility, and biofilm formation. Infection and immunity 77, 1543–1552.
102. Uhlich, G.A., Keen, J.E., and Elder, R.O. 2001. Mutations in the csgD promoter associated with variations in curli expression in certain strains of Escherichia coli O157: H7. Applied and environmental microbiology 67, 2367–2370.
103. Van Gerven, N., Van der Verren, S.E., Reiter, D.M., and Remaut, H. 2018. The role of functional amyloids in bacterial virulence. Journal of molecular biology 430, 3657–3684.
104. Vidal, O., Longin, R., Prigent-Combaret, C., Dorel, C., Hooreman, M., and Lejeune, P. 1998. Isolation of an Escherichia coli K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new ompR allele that increases curli expression. Journal of bacteriology 180, 2442–2449.
105. Wu, H., Moser, C., Wang, H.-Z., Høiby, N., and Song, Z.-J. 2015. Strategies for combating bacterial biofilm infections. International journal of oral science 7, 1–7.
106. Zhang, D., He, W., Tong, Q., Zhou, J., and Su, X. 2016. Multi-omics analysis on the pathogenicity of Enterobacter cloacae ENHKU01 isolated from sewage outfalls along the Ningbo coastline. Proteome science 14, 1–14.
107. Zhou, Y., Smith, D.R., Hufnagel, D.A., and Chapman, M.R. (2013) Experimental manipulation of the microbial functional amyloid called curli. In *Bacterial cell surfaces*. Springer, pp. 53–75.
108. Zogaj, X., Bokranz, W., Nimtz, M., and Römling, U. 2003. Production of cellulose and curli fimbriae by members of the family Enterobacteriaceae isolated from the human gastrointestinal tract. Infection and immunity 71, 4151–4158.

Figures

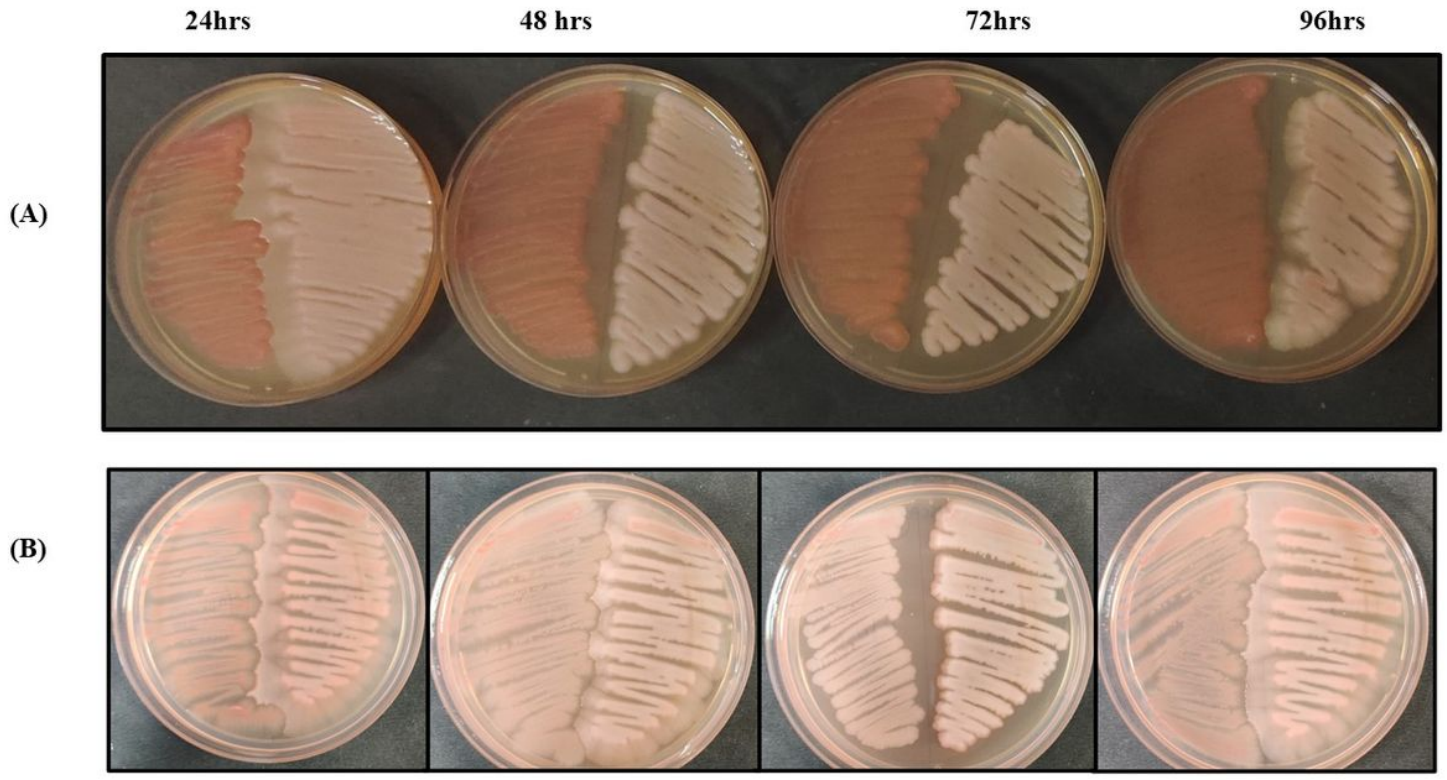

Figure 1

Congo red-containing yeast extract casamino acids (YESCA) plates comparing the Congo red binding phenotypes of wild type strain with the knockout strain. Panel (A) represents morphotypes grown at 25°C with varying time intervals of 24 to 96 hrs, and panel (B) represents morphotypes grown at 37° with varying time intervals 24 to 96 hrs.

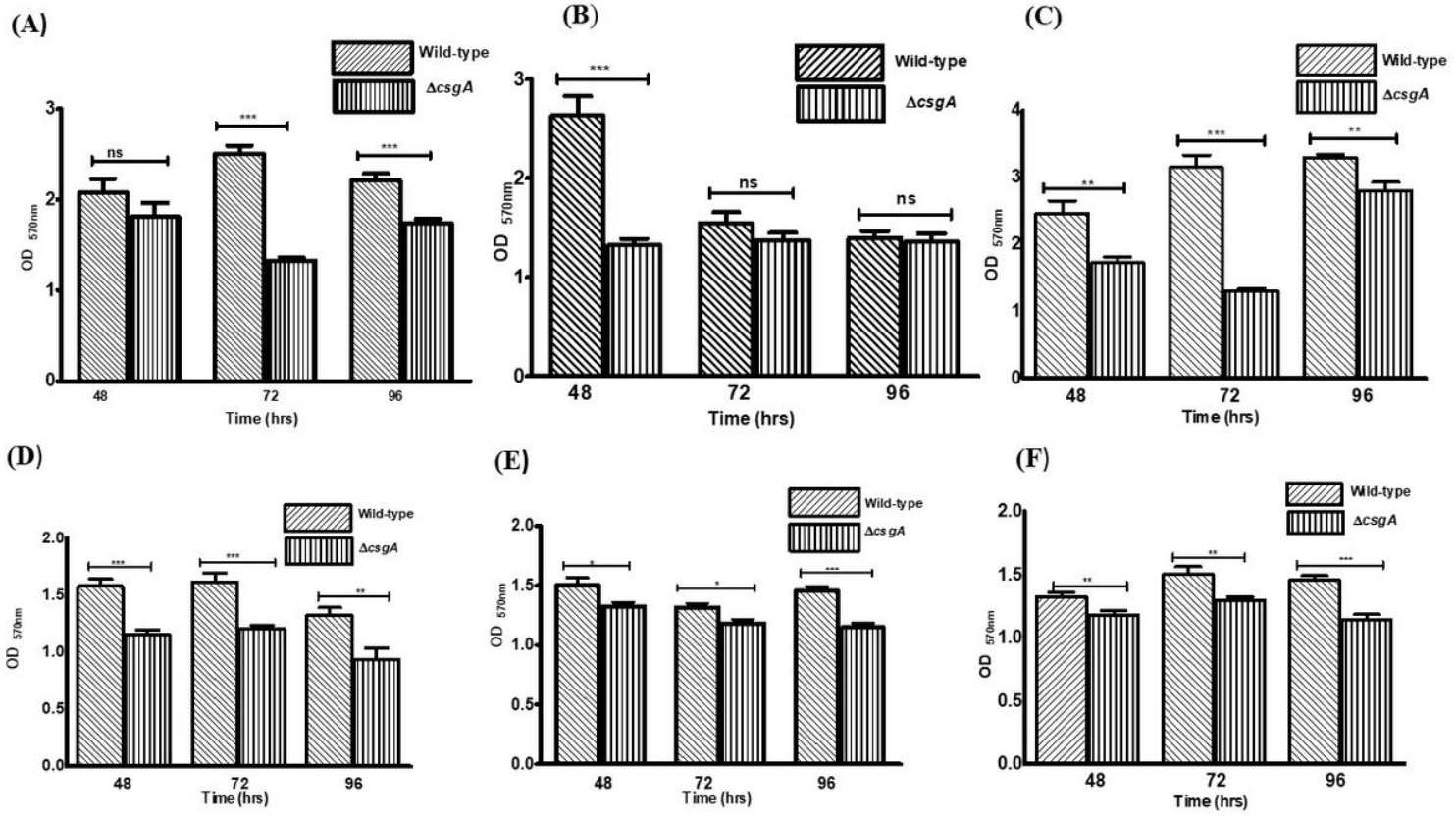

Figure 2

Crystal violet assay showing biofilm formation on different surfaces. Panels A-C shows biofilm formation at 25°C of surface of glass (A) Enteral feeding tube (B) and Foley latex catheter (C) Panels D-F shows biofilm formation at 37°C on surface of glass (D), Enteral feeding tube (E), and Foley latex catheter (F). Unpaired Student's t-test was performed for statistical significance: *, P≤ 0.05, **, P≤ 0.01, ***, P≤ 0.001, ****, P<0.0001, ns = not significant

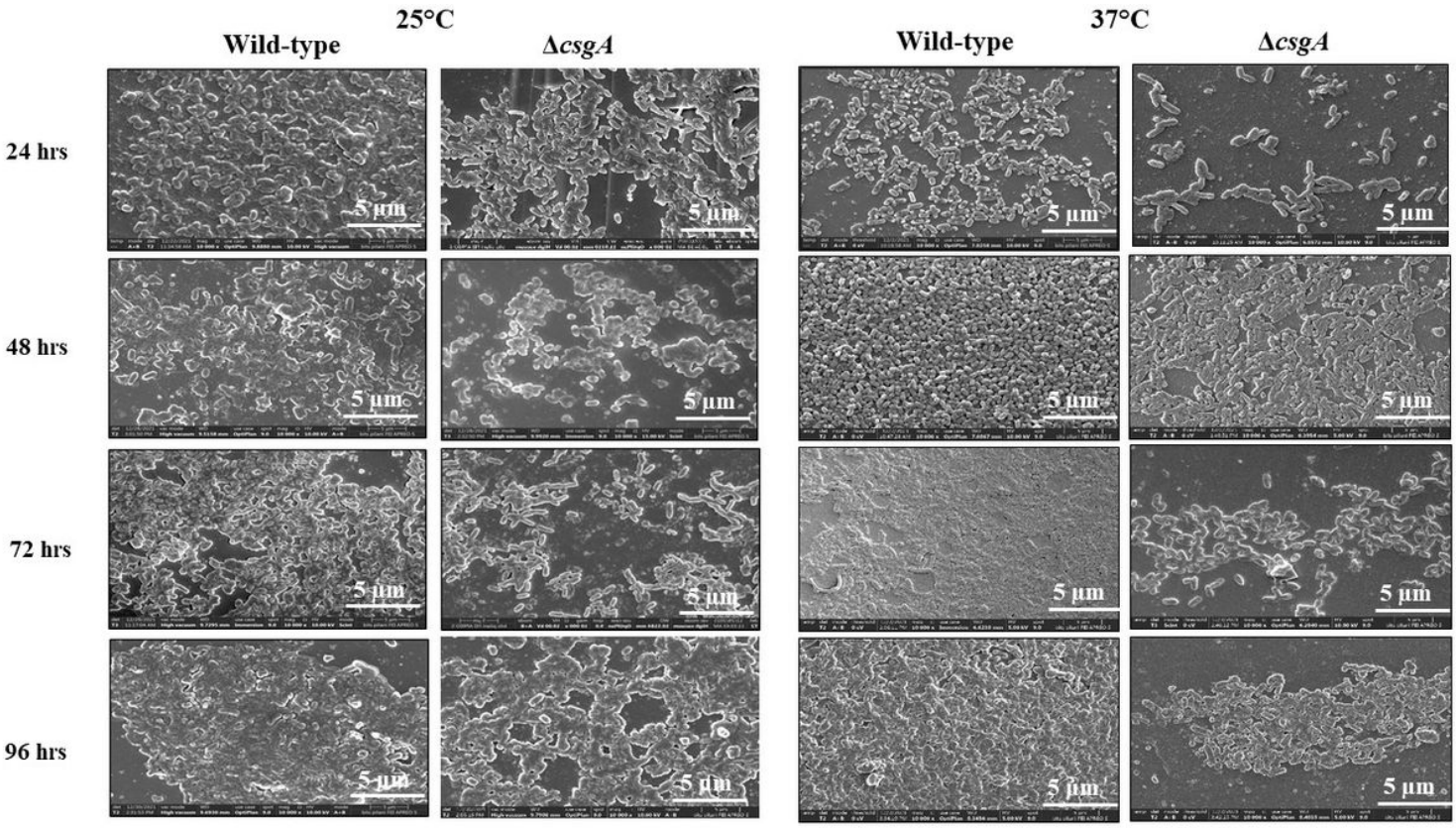

Figure 3

FESEM micrographs of the biofilm formed on the glass surface at 25° and 37°C by *E. cloacae* and its corresponding *csgA* mutant (10,000X). At different time points from 24-96 hrs , the wild-type strain adhered more on to the surface rather than the mutant strain.

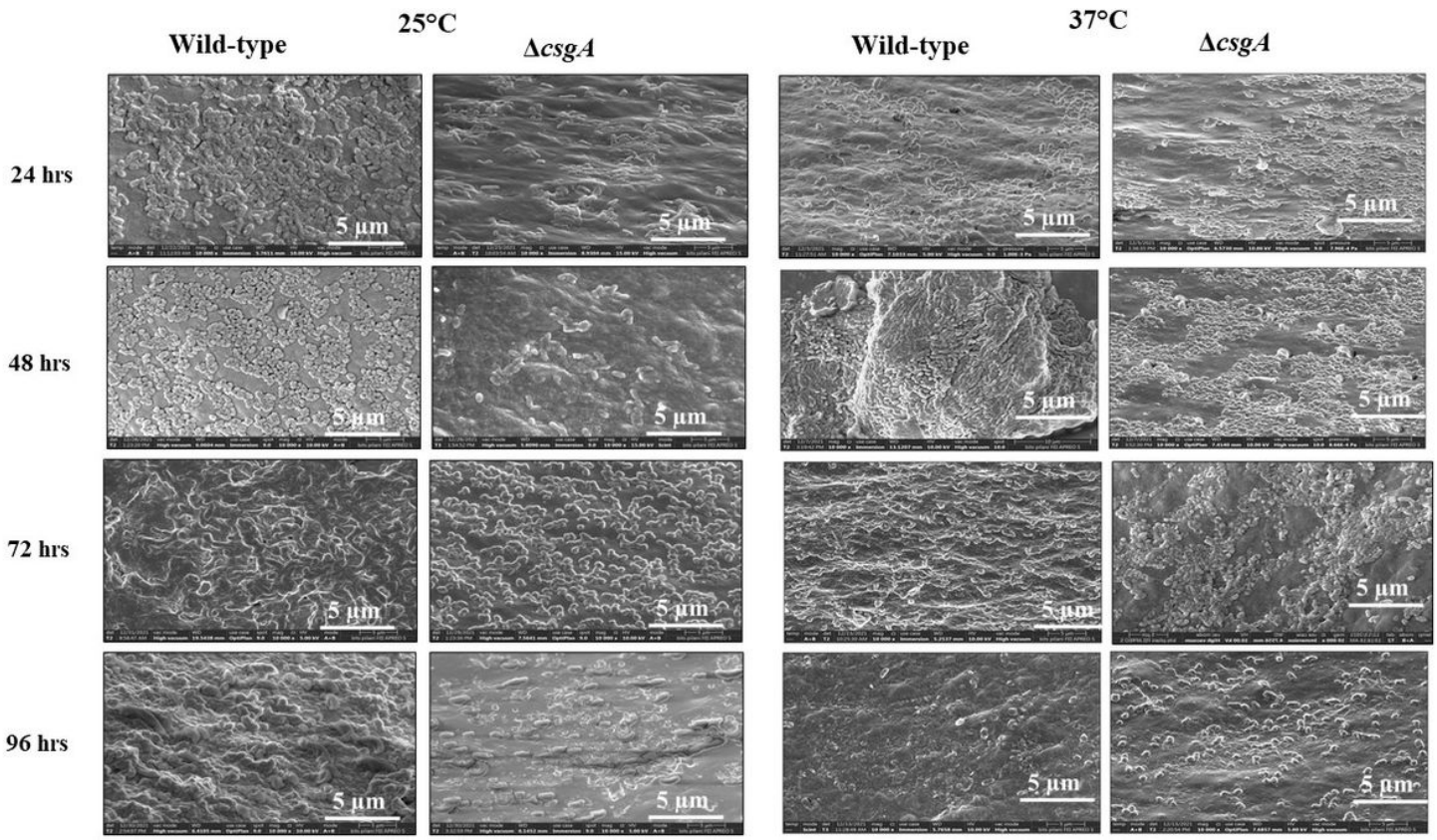

Figure 4

FESEM micrographs of the biofilm formed on the enteral feeding tube at 25° and 37°C by *E. cloacae* and its corresponding *csgA* mutant (10,000X). At different time points from 24-96 hrs , the wild-type strain adhered more on to the surface rather than the mutant strain.

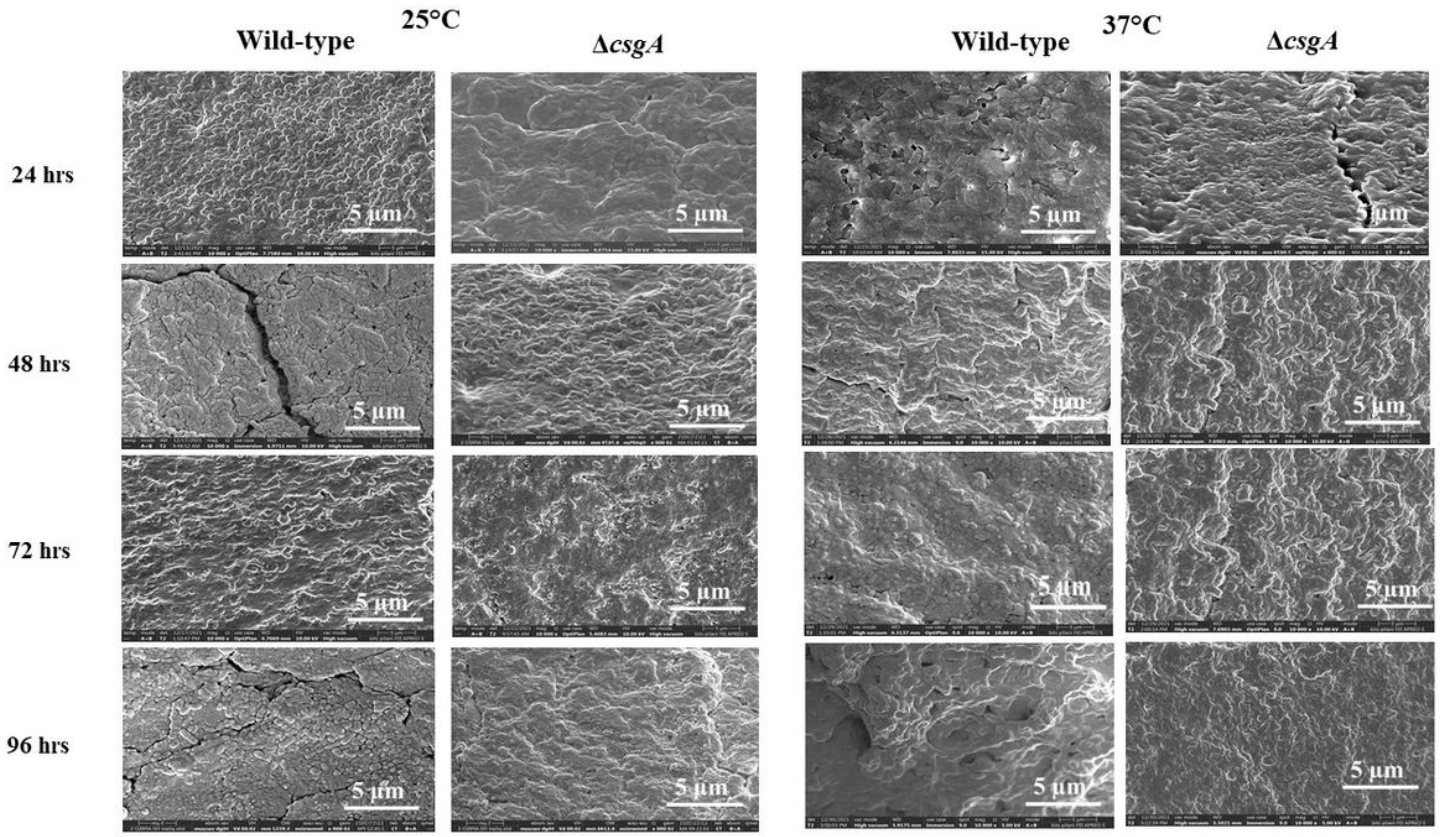

Figure 5

FESEM micrographs of the biofilm formed on the latex foley catheter at 25° and 37°C by *E. cloacae* and its corresponding *csgA* mutant (10,000X). At different time points from 24-96 hrs , the wild-type strain adhered more on to the surface rather than the mutant strain.

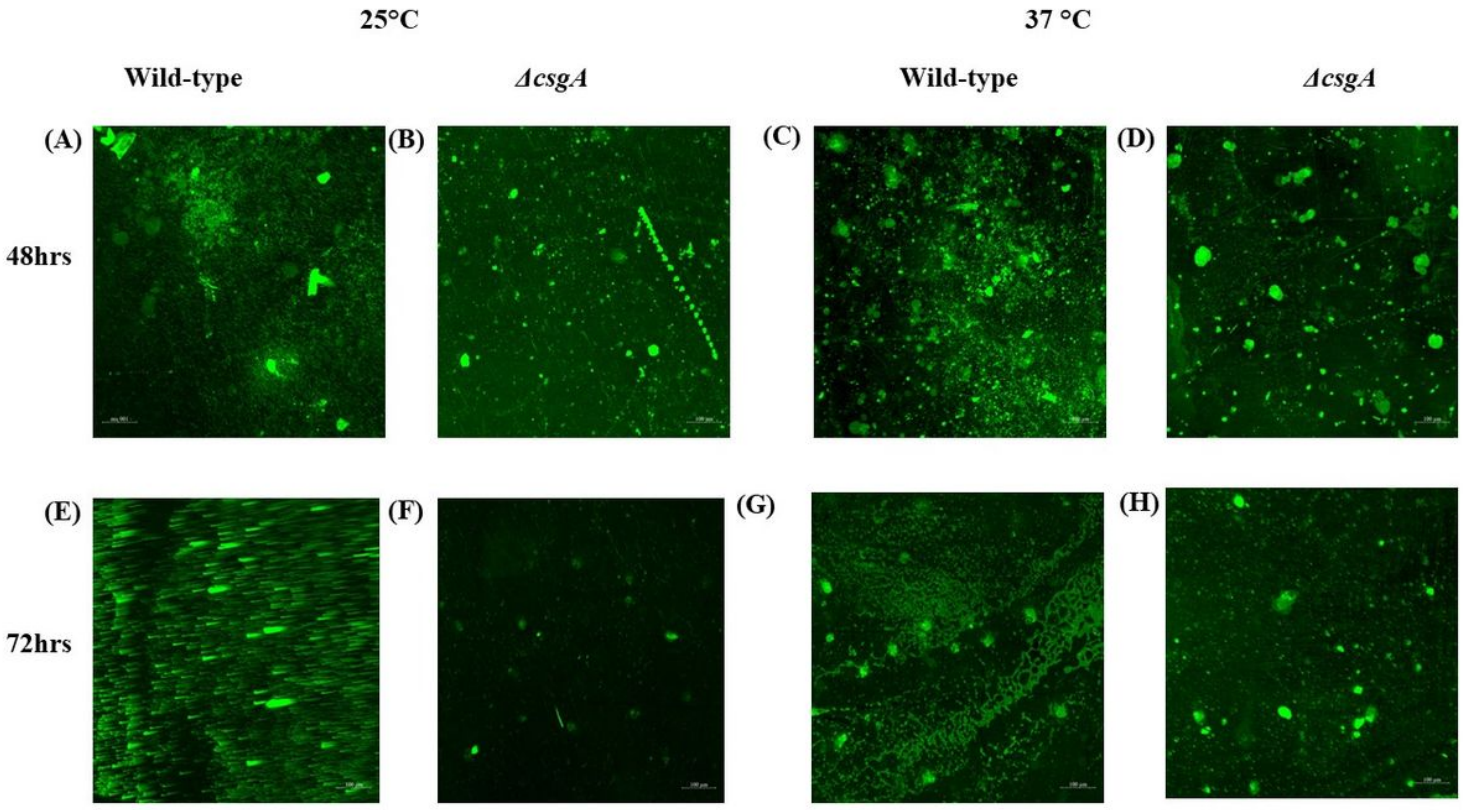

Figure 6

Confocal laser scanning microscopy images of biofilm formed by the wild-type and the mutant strain (*ΔcsgA*) incubated at 25°C and 37°C for 48 and 72 hrs under static conditions. All of these CLSM XY, or XZ-scan images were visualized with SYTO9 9 (green).(Scale :100μm) .Dense multi-layered biofilms were seen in the wild-type strains as depicted in (A),(C),(E),(G) Lesser cellular density with monolayer and micro-colony were seen in the mutant strain as depicted (B),(D),(F),(H).

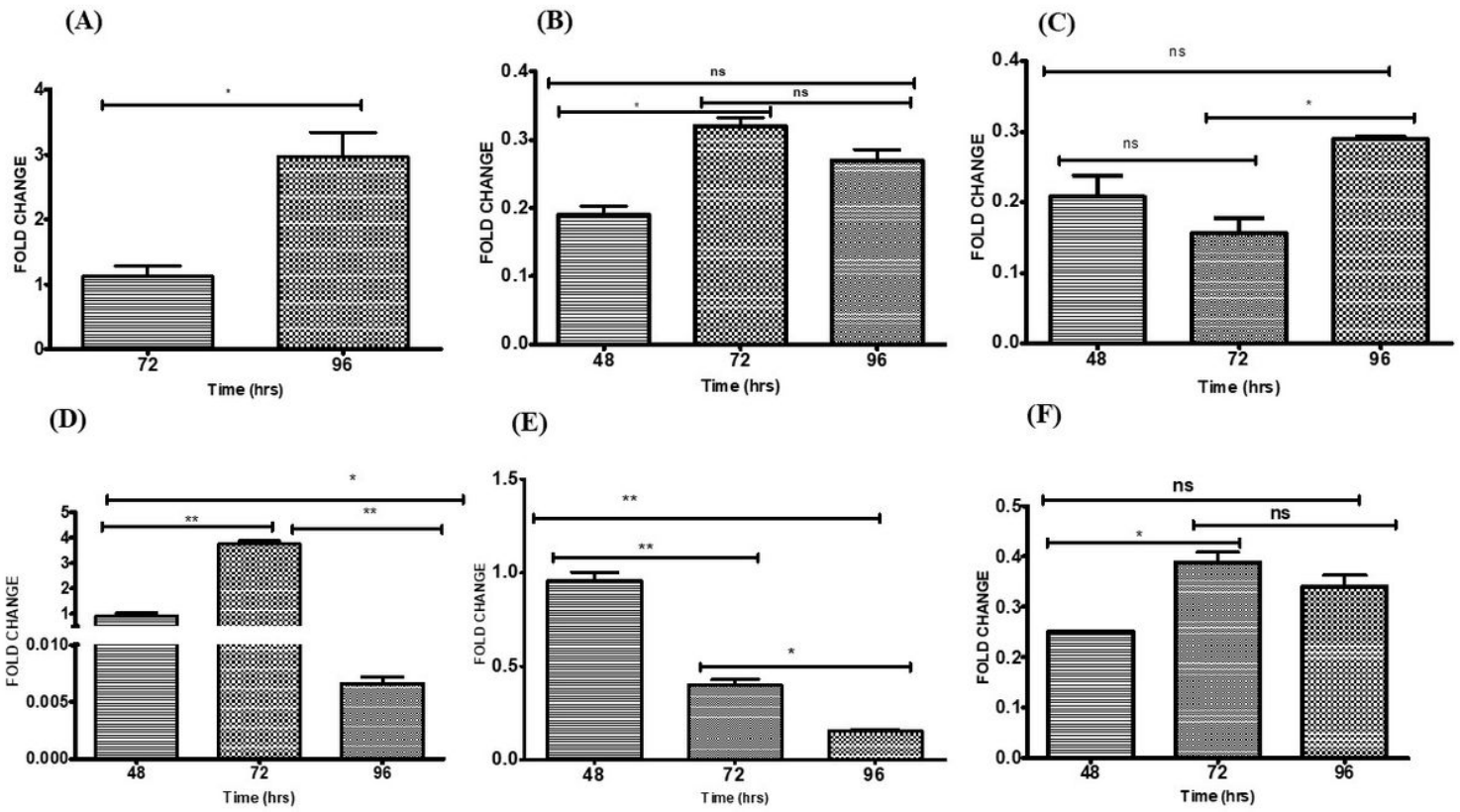

Figure 7

Curli gene expression at various surfaces. Panel A-C shows gene expression level of *csgA* at 25°C on Glass (A), Enteral feeding tube (B), and Foley latex catheter (C). Panels D-F shows *csgA* profile at 37 °C on at Glass (D), Enteral feeding tube (E), and Foley latex catheter (C)). Unpaired Student's t-test was performed. Statistical significance: *, P≤ 0.05, **, P≤ 0.01, ***, P≤ 0.001, ****, P<0.0001, ns = not significant

Supplementary Files

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