Gating curli formation: Three key residues in CsgE and their role in CsgE function

Sarah Kang
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Sponsor: Matthew R. Chapman, PhD
University of Michigan
Department of Molecular, Cellular, and Developmental Biology

University of Michigan – Ann Arbor
College of Literature, Science, and the Arts

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ABSTRACT

Curli are a class of functional amyloids, proteinaceous fibers secreted by many enteric bacteria. Curli help bacteria adhere to surfaces and colonize their hosts, and are an important component of biofilms, which increase bacterial resistance to antibiotics, desiccation, and other environmental stresses. The secretion and formation of curli fibers is a highly regulated process that is coordinated through the actions of many cellular proteins. The focus of my thesis is CsgE, a periplasmic protein that is thought to play a chaperone-like role in facilitating the secretion of the major curli subunit, CsgA, across the outer membrane. Purified CsgE has been shown to inhibit CsgA polymerization *in vitro*, suggesting that it helps prevent inappropriate amyloidogenesis in the cell. CsgE can also form a complex with CsgG, the outer membrane pore through which CsgA is secreted. CsgE has been shown to block the pore via some sort of gating mechanism, as demonstrated through erythromycin sensitivity assays. When CsgG alone is overexpressed, cells are susceptible to erythromycin as the antibiotic is able to enter the cell, but when CsgG is coexpressed with CsgE, erythromycin resistance is restored as CsgE plugs the outer membrane pore.

My thesis examines three point mutations in CsgE and how they impact CsgE function. Three mutants were constructed: a Trp-Ser mutation at the 49th amino acid (W49S), a Trp-Ser mutation at the 52nd amino acid (W52S), and a Phe-Ser mutation at the 68th amino acid (F68S). These mutants were then evaluated *in vivo* to determine their effects on curli formation and interactions with CsgG. I found that while the W49S and W52S mutants were still able to form curli fibers, the F68S mutant was not as able to form curli fibers but instead displayed an intermediate pink phenotype when plated on Congo red. Additionally, the F68S CsgE mutant

appeared in greater quantities when visualized on Western blot, suggesting that this mutant results a more stable protein configuration. Next, a series of erythromycin sensitivity assays were performed assessing the ability of each mutant to interact with CsgG via a gating or blocking mechanism. Whereas the W49S and W52S mutants did not confer erythromycin resistance, the F68S mutant was able to confer resistance similar to or even greater than wild type. Thus, the mutation at the 68 site may be affecting the stability of CsgE in a way that enhances its ability to block CsgG, but interferes with its ability to act as a chaperone in facilitating the secretion of CsgA. The W49S and W52S mutants, on the other hand, were unable to block the CsgG pore, suggesting that these sites are important in forming the CsgE-CsgG complex. These three sites offer promising insight into the crystal structure of CsgE and how the structure shapes its interactions with other proteins involved in curli secretion.

BACKGROUND AND INTRODUCTION

Amyloids are ordered, proteinaceous fibers characterized by the aggregation of peptides into β-sheet rich structures that are highly resistant to denaturation and digestion [1]. Protein misfolding and amyloid formation is the hallmark of several neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's diseases [2-4]. However, amyloid formation does not always arise as the product of protein misfolding. A specific subset of amyloids called "functional amyloids" are assembled by nearly all cell types and organisms [5, 6]. Bacterial functional amyloids play a critical role in surface attachment and biofilm formation [7-9]. Biofilms are useful to bacteria for a variety of reasons. The barrier formed by the extracellular polymeric secretions serves to trap antimicrobial agents, protecting the cells within [7]. Additionally, biofilms allow for the existence of pockets of "dormant zones" containing bacteria that are alive but not actively growing and dividing. Since most antibiotics are only effective in the presence of cellular activity, having dormant zones allows for the survival of bacteria that can then continue perpetuating the biofilm. Finally, it has been hypothesized that small populations of super-resistant bacteria can exist that contribute to the overall resistance of the biofilm [10]. Biofilm formation is a highly coordinated and regulated process, and understanding the genes and proteins involved in biofilm formation and persistence can thus offer insight to potential targets to their eradication.

Curli were the first identified functional amyloid and are produced by many enteric bacteria, including *Escherichia coli* and *Salmonella* spp [8, 9, 11]. Curli are non-branching proteinaceous fibers 4-12 nm wide, composed of a β-sheet rich structure that is resistant to denaturation and protease digestion [5]. They have been demonstrated to be the major

proteinaceous component of *E. coli* and *Salmonella* biofilms, with cellulose and polysaccharides representing the other biofilm components [11]. Curli are a useful and essential component of the biofilm, as curliated biofilms have been shown to have greater resistance to desiccation and treatment with hydrogen peroxide compared to non-curliated biofilms [12].

In *E. coli* biofilms, curli production is delegated to a specific subpopulation of cells at the air-colony interface [13]. Thus, curli secretion is a highly controlled process that is influenced by many environmental factors. In addition, many cellular regulators and accessory proteins are involved to ensure that curli formation takes place at the desired place and time. Figure 1 provides an overview of curli biogenesis. There are two divergently transcribed operons controlling curli secretion [14]. The first is the *csgBAC* operon, which encodes the major and minor curli subunits CsgA and CsgB respectively, as well as the periplasmic protein CsgC. CsgA, the major subunit, composes the curli fibers themselves. Like all amyloid fibers, purified CsgA is capable of self-polymerizing *in vitro*, and binds the amyloid-specific dyes Congo red and thioflavin T which allow for visualization of curli fibers [15]. CsgB, the minor curli subunit, is structurally similar to CsgA and nucleates fiber formation on the cell surface. CsgB from one cell is able to interact with soluble CsgA produced by other cells in a process called interbacterial complementation [16]. Both CsgA and CsgB are required for effective curli formation *in vivo*. CsgC is a small periplasmic protein that has been demonstrated to be a potent inhibitor of intracellular amyloid formation, and keeps the major and minor curli subunits from assembling inappropriately inside the cell [17].

The second operon required for curli secretion is the *csgDEFG* operon, which encodes several accessory proteins required for successful curli formation. CsgD is a transcriptional

regulator responsible for regulating both curli operons. Insertions in *csgD* completely abolish transcription from the *csgBAC* operon, suggesting that regulatory effects on the *csgDEFG* operon affect both operons [14]. CsgE, CsgF, and CsgG are all accessory proteins that play a role in mediating and facilitating the secretion of the major and minor curli subunits across the outer membrane. CsgG is a lipoprotein that is localized to the outer membrane and facilitates the diffusion of CsgA out of the cell. Analysis of the crystal structure reveals that its configuration in the membrane consists of a cage-like β-barrel which permits the diffusion of peptides [18]. When overexpressed, CsgG can also act as a nonspecific channel through which periplasmic proteins can diffuse out of the cell or small molecules such as antibiotics can enter the cell [18, 19]. It has been shown that overexpression of CsgG leads to erythromycin sensitivity [19].

CsgE and CsgF are chaperone-like proteins that work with CsgG to help facilitate the secretion of the curli subunits. Cells with a *csgF* deletion are still able to secrete soluble CsgA and donate CsgA to nearby cells for complementation, but are unable to form their own curli fibers. Thus, it is thought that CsgF is involved in the nucleation step of fiber formation, which takes place on the outer membrane. CsgF plays a role in the proper localization of CsgB and in mediating CsgB protease resistance [20].

Whereas CsgF is localized to the outer membrane, CsgE remains inside the periplasm and is thought to play a chaperone-like role in coordinating the secretion of the major curli subunits through CsgG. Cells with a *csgE* deletion are defective for curli secretion and assembly [19]. Like CsgC, purified CsgE is able to inhibit CsgA polymerization *in vitro*, suggesting that it helps prevent inappropriate amyloidogenesis in the cell [19]. In addition to interacting with

CsgA, CsgE is also capable of forming a complex with CsgG that gates the pore [18, 19]. At saturated concentrations of CsgE, the pore is completely blocked, while at concentrations of 1 nM or below, only transient interactions occur [18]. Thus, whereas overexpression of CsgG leads to erythromycin sensitivity, overexpression of CsgE confers erythromycin resistance through the blocking of the CsgG pore [19]. The exact mechanism by which CsgE blocks or gates CsgG is still uncertain, but this interaction likely plays a role in facilitating the secretion of CsgA.

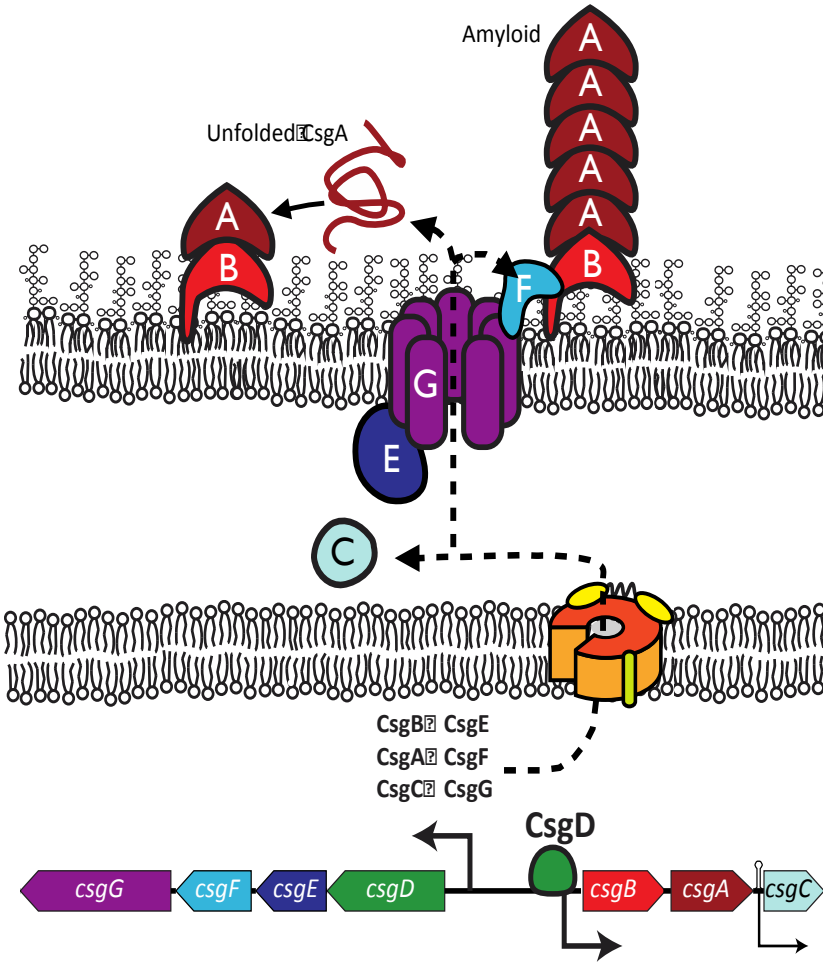

Figure 1: Overview of curli biogenesis. All Csg proteins (excluding CsgD) have Sec-dependent signal sequences. CsgE and CsgC are periplasmic proteins required for efficient curli assembly. The major subunit protein (CsgA) and the nucleator (CsgB) are secreted to the cell surface in a CsgG/E-dependent fashion. CsgB associates with the outer membrane (and CsgF) where it nucleates soluble, CsgA into a highly ordered amyloid fiber.

My thesis focuses on CsgE and how mutations at three distinct sites affect the function of CsgE *in vivo*. CsgE is composed of 109 amino acids; of these, three have been identified as sites of interest thought to play an important role in its function and interactions with other proteins involved in curli secretion such as CsgG. Figure 2 illustrates these sites in the context of the 3-D structure of CsgE. The goal of these experiments was to create point mutations at each of these sites of interest and then evaluate the mutants to see how they affect CsgE function *in vivo.* The three mutants were a tryptophan to serine mutation at the 49th amino acid (W49S), a tryptophan to serine mutation at the 52nd amino acid (W52S), and a phenylalanine to serine mutation at the 68th amino acid (F68S). First, the ability of each of these mutants to complement curli formation was assessed through plating on Congo red. Western blots were carried out to compare levels of CsgG, CsgE, and CsgA between the mutants and wild type curli-producing cells. Finally, a series of erythromycin sensitivity assays were performed to assess the ability of each mutant to interact with CsgG.

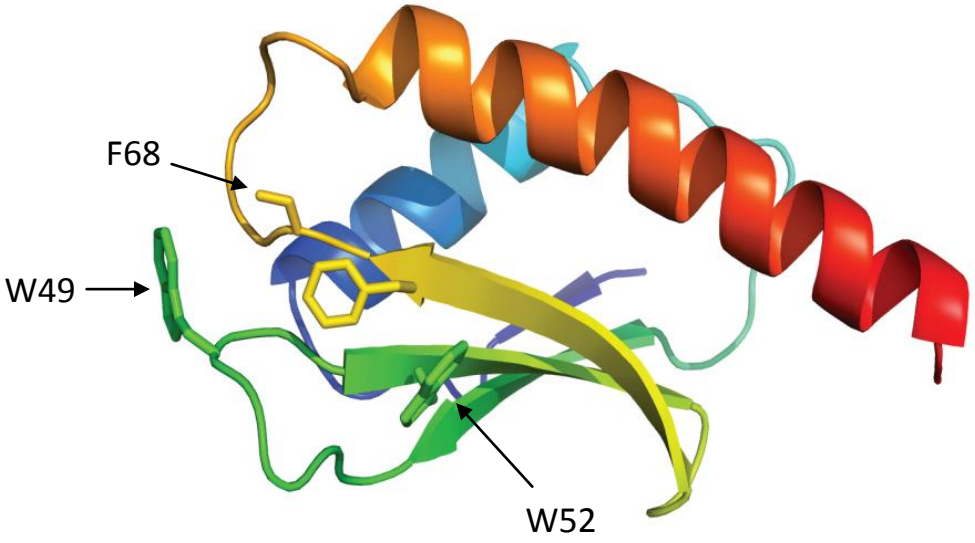

Figure 2: Model of CsgE as solved by our collaborator, Christiane Ritter (unpublished results). The approximate location of each site is indicated.

MATERIALS AND METHODS

csgE mutants

Three sites in *csgE* were assessed, and point mutations created at each. The first is at the 49th amino acid and consists of a tryptophan to serine mutation, thereafter referred to as W49S. The second is at the 52nd amino acid and consists of a tryptophan to serine mutation (W52S). The third is at the 68th position and consists of a phenylalanine to serine mutation (F68S). Mutations were introduced by fusion PCR and confirmed by sequencing.

Strain construction

E. coli strain LSR11 is derived from parent strain MC4100, which is a laboratory strain of *E. coli* that is wild type for curli-producing genes. LSR11 is a *csgE* mutant that was created by removing the *kan^R* cassette from LSR5 (MC4100 *Δcsg::kan^R*) [19].

Fusion PCR

Fusion PCR was used to introduce the desired mutations into pLR12, a plasmid encoding both *csgG* and *csgE*. Custom primers were designed containing the desired mutation in *csgE*. The first round of PCR paired one of the mutation-containing primers with an outer *csgE* primer located just outside the *csgE* gene, resulting in a gene fragment containing the desired mutation. The second round of PCR took the fragments from the first round of PCR and incubated them with both outer primers, thus amplifying the entire gene now containing the mutation. The PCR products were then digested with BamHI and NcoI and ligated into pTrc99A, an empty vector. This plasmid was then transformed into *E. coli* strain LSR11.

Congo red plating

First, *csgE* was cloned into pLR2, which contains the *csgBAC* promoter, allowing *csgE* to be expressed under normal curli-inducing conditions. These plasmids were then transformed into strain LSR11. Congo red YESCA plates were prepared using 10g/L CAS amino acids, 1g/L yeast extract, and 20g/L agar. 200:1 Congo red stock was added when the plates were cooled. 4 µl of cells normalized to 1 OD_{600} were then dotted onto the plates, streaked, and allowed to grow at 26°C for 48 hours.

Western blot

For CsgA: Cells were collected after being grown on LB plates at 37°C overnight, suspended in water, and normalized to 1 OD_{600}. For CsgA, treatment with formic acid or hexafluoroisopropanol (HFIP) is required to denature the fibers into monomers that can be resolved on an SDS-PAGE gel [15]. Thus, for each sample being blotted, two tubes were prepared: one for treatment with HFIP and the other to be untreated as a negative control. To treat with HFIP, the pelleted cells were resuspended in 75 µl HFIP, incubated at room temperature for 10 minutes, then the HFIP was dried in a vacuum centrifuge. Once the HFIP was completely evaporated, the cells were then resuspended in 40 µl 2X SDS buffer. The untreated cells were simply pelleted and resuspended in 40 µl 2X SDS buffer. All the samples were then boiled at 100°C for 10 minutes before being run on an SDS-PAGE gel. Protein was then transferred onto a PVDF membrane and blotted with primary antibody and a second fluorescent antibody.

For CsgG/CsgE: For CsgG and CsgE, no treatment with formic acid or HFIP is necessary. Cells were collected after being grown on LB plates at 37°C overnight, normalized to 1 OD_{600},

and resuspended in 40 µl 2X SDS buffer. Samples were then boiled at 100°C for 10 minutes before being run on an SDS-PAGE gel. Protein was then transferred onto a PVDF membrane and blotted with primary antibody and a second fluorescent antibody.

Erythromycin sensitivity assay

E. coli strain LSR11 containing pTrc99A (empty vector), PMC1 (WT CsgG), pLR12 (WT CsgG and WT CsgE), and plasmids containing each of the three mutations were used in this assay. Cells were grown overnight in LB, then diluted 1:100 into 10mL LB and grown at 37°C with shaking. Expression was induced with 0.05mM IPTG at 0.5 hr, and then 10 µg/ml erythromycin was added at 1 hr [19]. Optical density was measured once every 0.5 hour for 4 hours. Optical density was measured using a Klett meter, which provides OD in Klett units.

RESULTS

Observing how these mutations affect CsgE function *in vivo* offers insight to how these sites function in the context of the cell. In order to better understand how these three residues contribute to CsgE function, point mutations were created at each position and then the mutant *csgE* sequences cloned into pLR2, which contains the *csgBAC* promoter. This allows the mutant forms of CsgE to be expressed under normal curli-inducing conditions. The plasmids containing WT or mutant CsgE were then transformed into LSR11, a *csgE* deletion strain, so that their ability to complement curli formation could be assessed.

The first step was to qualitatively assess how these mutations affected curli production by plating them on Congo red and observing the Congo red binding phenotype [16]. Congo red is an amyloid-specific dye that binds to polymerized fibers. MC4100 (WT curli-producing cells) appears red, whereas LSR11 (MC4100 *csgE*-), which is defective for curli production, appears white (Figure 3). The *csgE*- mutant complemented with either the W49S or W52S CsgE mutants displayed a red phenotype comparable to complementation with wild type CsgE (Figure 3A-C). However, complementation with the F68S CsgE mutant displayed an intermediate pink phenotype (Figure 3D), indicating that there was a defect in curli biogenesis when the F68S CsgE mutant protein was expressed.

To better quantify the protein levels in each strain, Western blots were performed using antibodies specific against CsgA (the major curli subunit), CsgG (outer membrane secretion protein) and CsgE. CsgG levels remained fairly uniform between all the strains and plasmids assessed (Figure 4A-F). CsgE levels were measured using the α-CsgE antibody where CsgE was expressed from a plasmid. Note that the α-CsgE antibody is unable to recognize CsgE expressed

natively from the chromosome, possibly due to low levels of endogenous CsgE [21]. The W49S mutant and WT CsgE proteins accumulated to approximately the same levels (Figure 4CD). The W52S mutant appeared to have slightly elevated CsgE levels (Figure 4E), and the F68S mutant had significantly higher levels than WT CsgE (Figure 4F), suggesting that these mutations alter the steady state levels of CsgE. Polymerized CsgA fibers are resistant to denaturation, and must be treated with a strong denaturant such as hexaflurorisopropanol (HFIP) in order to be resolved on SDS-PAGE gel [15]. Figure 4 shows HFIP-treated samples alternating with untreated samples. Interestingly, CsgA levels for the W49S and W52S mutants were comparable to wild type (Figure 4C-E), although the F68S mutant had considerably less CsgA (Figure 4F).

Following the Congo red plating and initial Western blots, it was apparent that the F68S mutant was unique from the other two mutants. The increased amounts of CsgE present on the Western blot suggest that the F68S mutation is causing the protein to take on a more stable configuration. However, it was unclear whether the decrease in curli fibers seen on Congo red was due to changes in how the F68S mutant was interacting with CsgG or CsgA. Thus, in order to gain a better understanding of how the CsgE mutants are able to gate CsgG, a series of erythromycin sensitivity assays were performed.

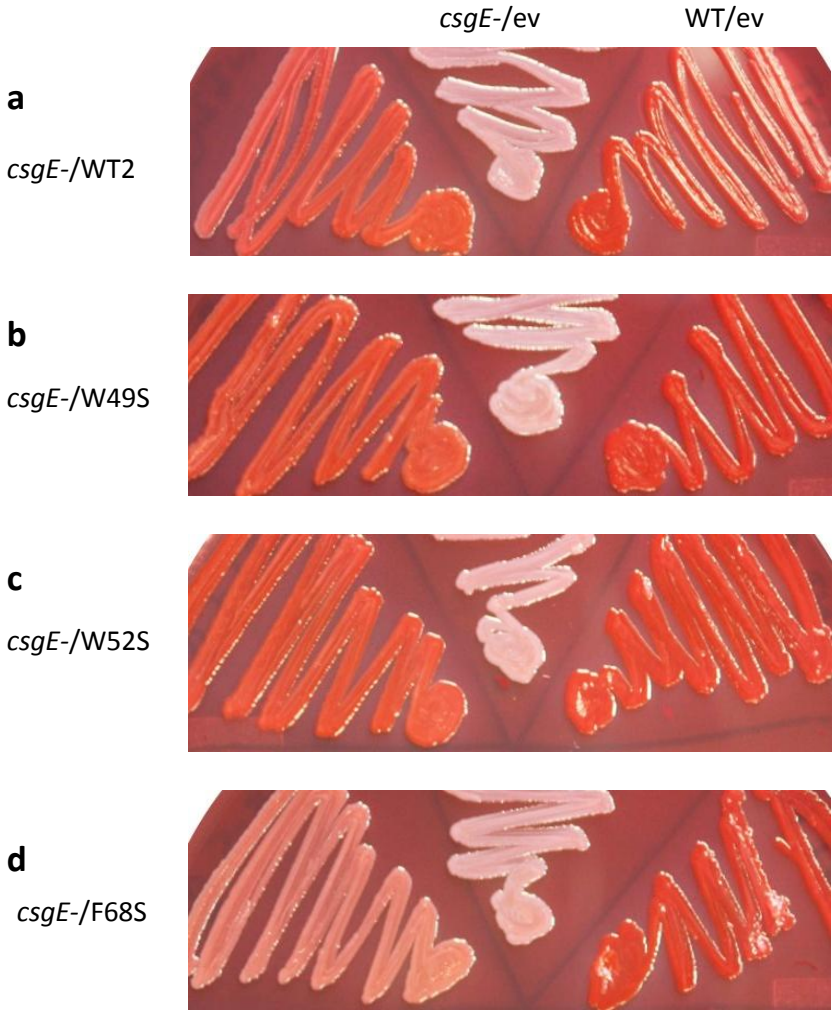

Figure 3: Congo red binding phenotype of *csgE* mutants compared to wild type (MC4100) and cells containing a *csgE* deletion (LSR11). Cells were streaked onto Congo red plates and grown at 26°C for 48 hours. MC4100 produces a bright red phenotype, whereas LSR11 has a white phenotype. **a-c)** The W49S and W52S mutants display a red phenotype comparable to the wild type. **d)** The F68S mutant, however, displays an intermediate pink phenotype.

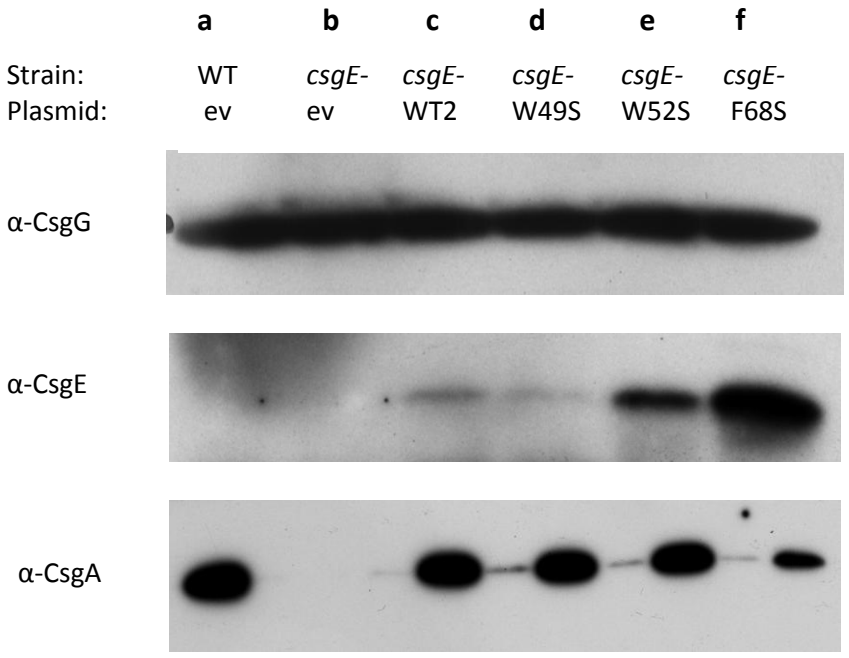

Figure 4: CsgG, CsgE, and CsgA levels visualized by Western Blot. Cells were grown overnight on LB plates, collected and normalized to 1 OD_{600}. To visualize CsgA, samples were treated with HFIP before being resuspended in SDS buffer, boiled, and run on an SDS-PAGE gel. CsgG and CsgE samples were simply resuspended in SDS buffer, boiled, and run on SDS-PAGE. CsgG levels are fairly uniform between wild type and the three mutants. For CsgE, the W49S mutant has protein levels similar to WT2 **(d)** (note that the α-CsgE antibody is unable to bind to CsgE from the wild type strain). TheW52S mutant has slightly more protein **(e)**, and the F68S mutant has significantly more **(f)**, indicating that these mutants may have a more stable configuration. CsgA levels are fairly uniform between wild type and the first two mutants, while the F68S mutant displays significantly less CsgA. Western blots were replicated at least three times with very similar results.

The purpose of the erythromycin sensitivity assays was to evaluate the ability of the CsgE mutants to interact with CsgG via a gating mechanism. In order to allow CsgE and CsgG expression to be induced at the same time, the desired mutations were introduced into *csgE* via fusion PCR, and then both the *csgE* mutants and *csgG* were cloned into pTrc99A under the same promoter. The erythromycin sensitivity assays were carried out on LSR11 with pTrc99A (empty vector), pMC1 (WT CsgG only), pLR12 (WT CsgG and WT CsgE), and then plasmids carrying the mutant constructs in pTrc99A. The empty vector was mostly unaffected by the erythromycin, whereas cells expressing CsgG only were susceptible to erythromycin and died off quickly (Figure 5AB). The coexpression of WT CsgE with CsgG conferred erythromycin resistance (Figure 5C). The W49S and W52S mutants were highly susceptible to erythromycin and did not confer resistance (Figure 6AB). Their growth curves resemble that of CsgG alone (Figure 5B). The F68S mutant, however, did confer erythromycin resistance, and even slightly more so than WT CsgE (Figures 5C and 6C).

Western blots were also performed on the cells that underwent the erythromycin sensitivity assays in order to ensure that both CsgE and CsgG were being expressed, and to evaluate their levels in the cell. CsgG levels were uniform among all the cells (except the empty vector). CsgE levels were fairly uniform between the WT CsgE and W49S and W52S mutants that were not treated with erythromycin, although the F68S mutant again displayed slightly elevated levels. For the cells that were treated with erythromycin, a faint band of CsgE is visible for the wild type and F68S mutant, but no CsgE is visible for the W49S and W52S mutants (Figure 8).

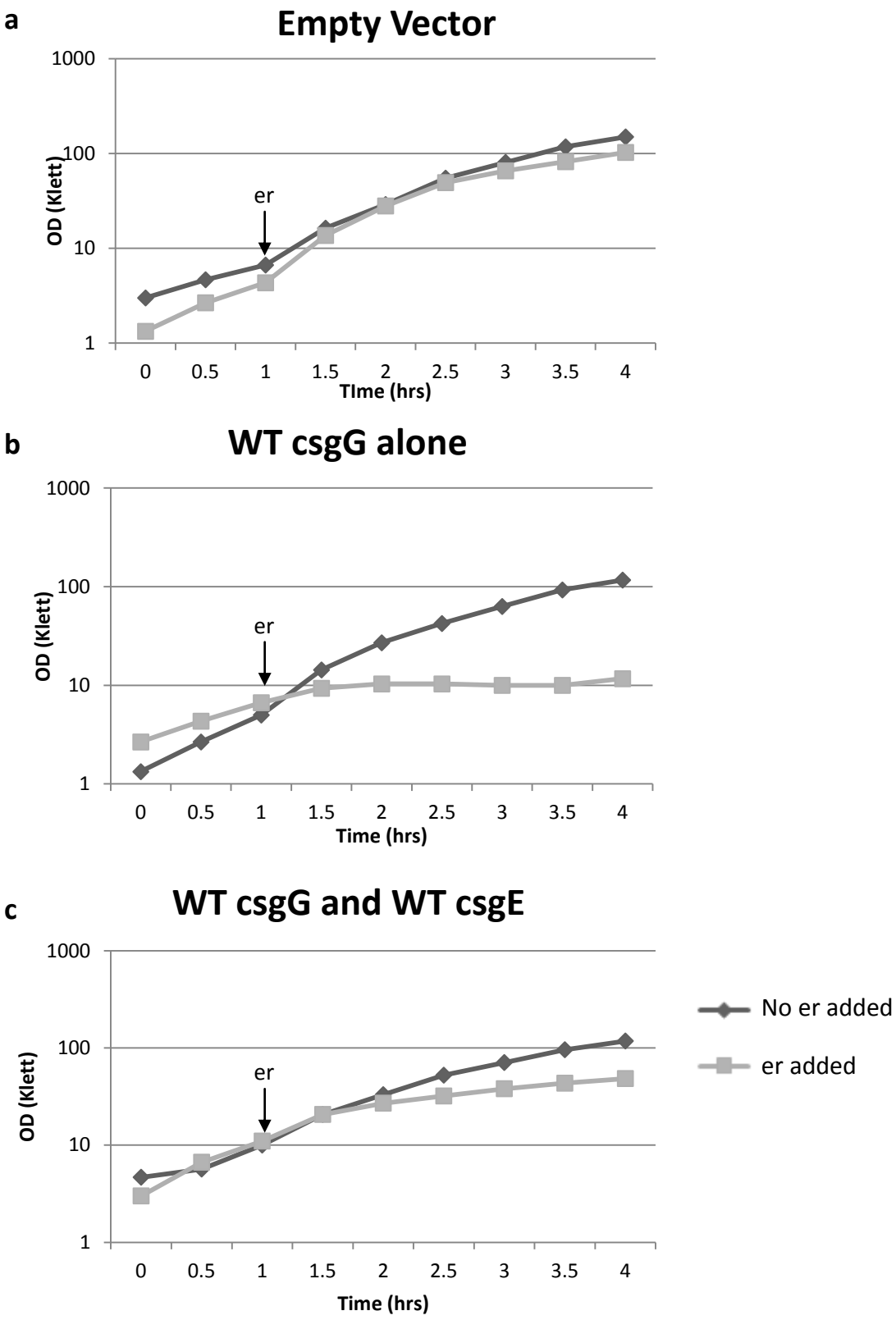

Figure 5: Erythromycin sensitivity assay controls. **a)** Empty vector, which expresses neither CsgG nor CsgE, is mostly unaffected by erythromycin. **b)** Cells overexpressing CsgG are highly susceptible to killing by erythromycin. **c)** Cells expressing both CsgG and CsgE experience erythromycin resistance.

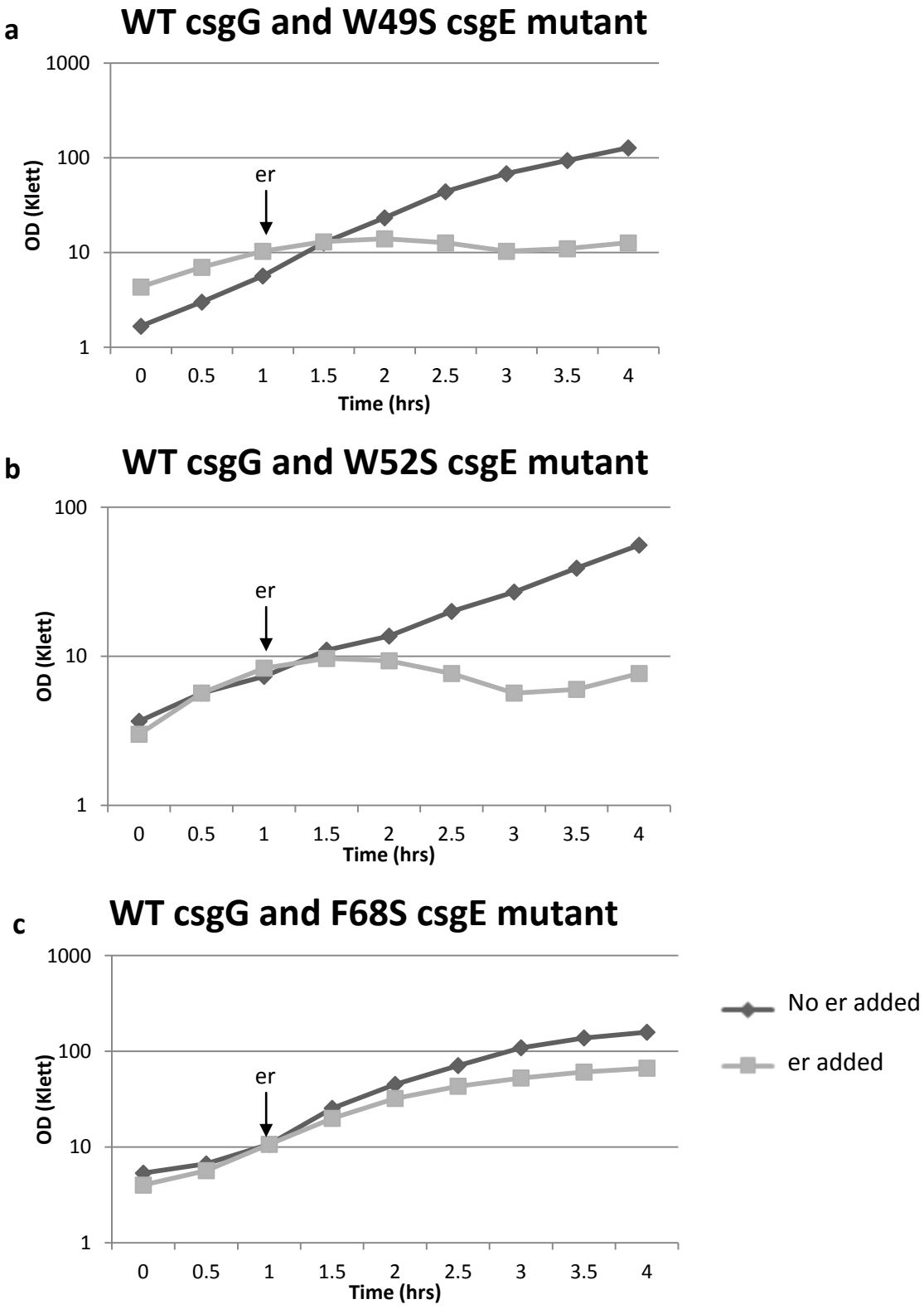

Figure 6: Erythromycin sensitivity assay with CsgE mutants. **a-b)** Expression of the W49S and W52S mutants do not confer resistance. **c)** However, expression of the F68S mutant does confer erythromycin resistance.

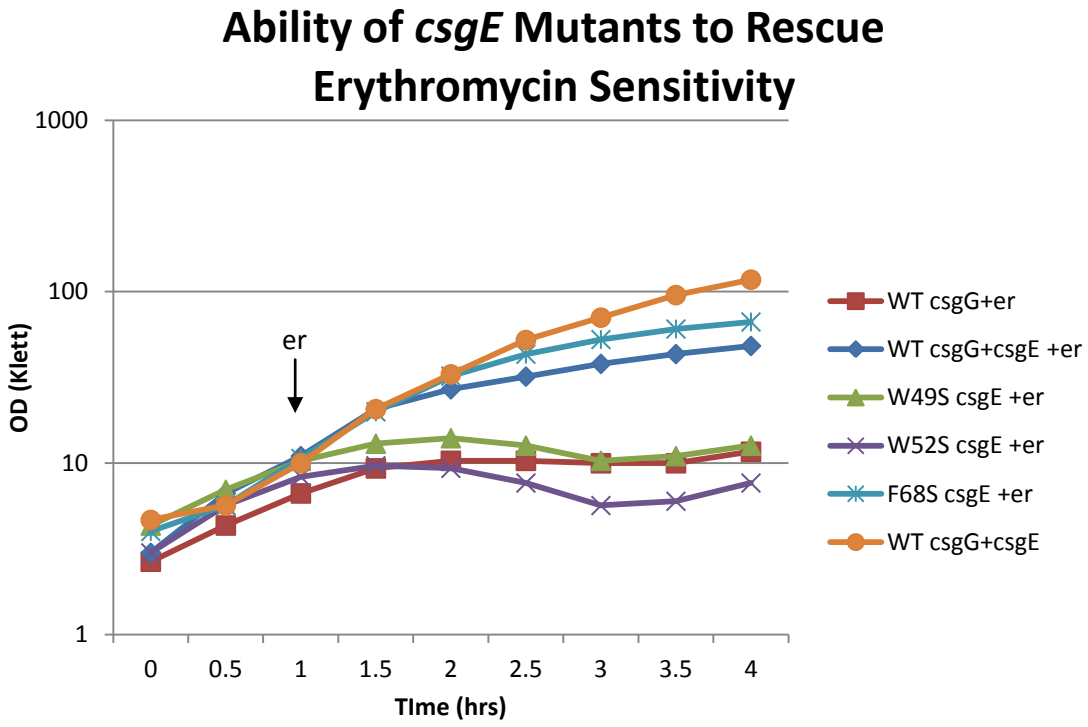

Figure 7: Summary of erythromycin sensitivity assays showing rescue ability of mutants compared to WT CsgG alone, and WT CsgG and CsgE with and without erythromycin. The F68S mutant (light blue) confers slightly more resistance than wild type (dark blue). The W49S and W52S mutants (green and purple) are approximately as susceptible as CsgG only (red).

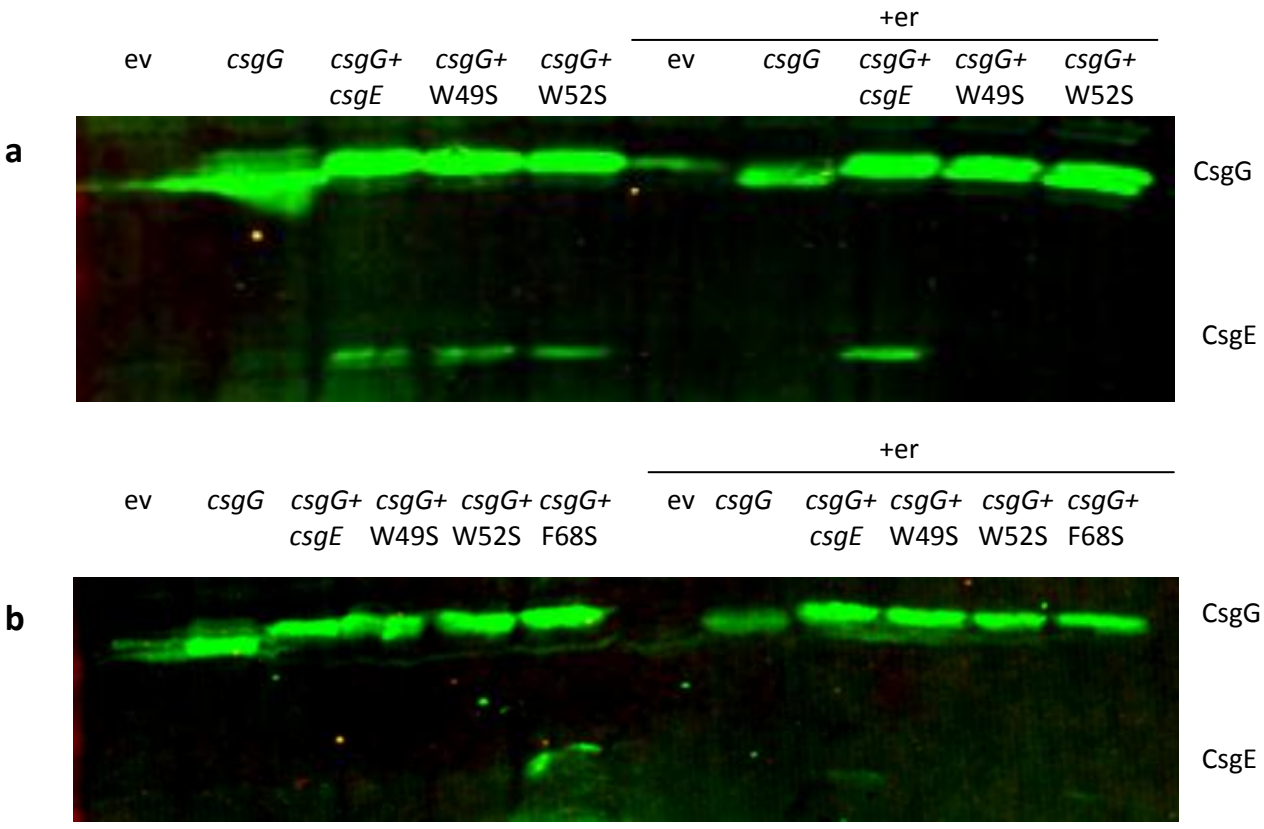

Figure 8: CsgG and CsgE levels in cells after growth curve. Cells were collected after being grown with or without erythromycin treatment for four hours, normalized to 1 OD600, then pelleted and run on SDS-PAGE gel. CsgE is clearly present in the cells that were not treated with erythromycin, but are not present in the W49S and W52S strains that were treated with erythromycin **(a)**. Once again, increased CsgE levels are seen in the F68s mutant. A faint band is visible for the F68S treated with erythromycin whereas no CsgE is present for the W49S and W52S mutants **(b)**.

DISCUSSION

Among the three mutants, the W49S and W52S mutants appear to result in relatively similar results for each assay performed, whereas the F68S mutant is markedly different. This was apparent in the Congo red binding phenotype of each mutant, where complementation with the W49S and W52S mutants displayed a Congo red phenotype similar to complementation with wild type plasmid (Figure 3A-C), and complementation with the F68S mutant displayed a unique intermediate phenotype (Figure 3D). This intermediate pink phenotype suggests that curli secretion and assembly is not completely inhibited, yet the fibers forming are certainly not as robust as wild type. Thus, interfering with the 68 site in CsgE impairs its ability to function in the secretion of CsgA, yet not to the degree of a *csgE* deletion mutant.

There are several hypotheses that could explain the F68S mutant's impaired curli formation. Firstly, this site could be important for the interactions between CsgE and CsgG, and a mutation at this site is causing it to block the pore in a way that does not allow CsgA through. Since F68S CsgE displayed greater stability as visualized by Western blot (Figure 4F), perhaps the increase in stability increases the favorability of its configuration with CsgG, thus clogging the pore and decreasing CsgA secretion. Another hypothesis is that the 68 site is important for its interactions with CsgA, and a mutation here is impairing the affinity of CsgE with CsgA, and its ability to act as a chaperone. As for the W49S and W52S mutants, even though they displayed Congo red binding comparable to wild type, it is uncertain just from the Congo red plating whether or not they are still able to function like WT CsgE in the gating mechanism with CsgG.

The erythromycin sensitivity assays were conducted to assess how the CsgE mutations affect their interaction with CsgG. It has been established that overexpression of CsgG leads to erythromycin sensitivity, whereas CsgE expression is able to restore resistance by gating the pore [19]. Thus, the "rescue" ability of CsgE to confer erythromycin resistance provides insight to the gating interaction that takes place between the two proteins. Figure 5BC demonstrates this rescue ability by comparing overexpression of CsgG alone to coexpression with WT CsgE.

These assays were performed with plasmids expressing both WT CsgG and the mutant forms of CsgE (Figure 6A-C). The W49S and W52S mutants do not confer erythromycin resistance; the cells die off quickly upon exposure to erythromycin and the growth curves resemble those of CsgG alone. The F68S mutant, however, does confer erythromycin resistance, conferring a rescue effect even slightly greater than that of wild type (Figure 7).

Since the F68S mutant is able to confer erythromycin resistance, it appears that the gating mechanism with CsgG is not impaired by the mutation. In fact, since the F68S displayed slightly more rescue ability than WT CsgE, it is possible that the gating is even more long-lived or robust than with the wild type. This evidence supports the hypothesis that the F68S mutant is able to block the CsgG pore, but perhaps is doing so in a way that does not allow CsgA through effectively. The W49S and W52S mutants, on the other hand, displayed no rescue ability, suggesting that these mutations interfere with CsgE's ability to interact with CsgG. If these mutations decrease the affinity of CsgE with CsgG or otherwise hinder them from forming a complex, then the CsgG pore would remain ungated, allowing erythromycin to enter the cell. The increased permissivity of the pore would also permit CsgA to diffuse out of the cell, resulting in the robust Congo red binding phenotype (Figure 3BC).

Western blot was also performed on the cells that underwent the erythromycin assays in order to evaluate the levels of CsgG and CsgE. CsgG levels were fairly uniform between all the strains evaluated. CsgE levels were uniform for the cells that were not treated with erythromycin, but the W49S and W52S mutants that were treated with erythromycin had significantly lower levels of CsgE (Figure 8). This is likely due to the fact that these mutants were susceptible to erythromycin, and the cells were collected at the end of the assay when they had been exposed to erythromycin for 3 hours. Erythromycin acts by interfering with protein synthesis [22], thus, the erythromycin-killed cells would be unable to synthesize more CsgG and CsgE. CsgG, which remains in the membrane, would be intact even after pelleting the cells and appears on the Western Blot. CsgE, on the other hand, would be in the supernatant after pelleting, and does not show up in the Western. A faint band is visible for CsgE for the wild type and F68S cells, which were resistant to erythromycin.

All three of these sites have been shown to play important roles in the function of CsgE, as examined through Congo red binding, Western blot, and erythromycin sensitivity assays. The W49S and W52S mutants were not able to block the CsgG pore, yet were able to complement to form robust curli fibers, suggesting that these residues are important in the CsgE-CgG interaction. The F68S mutant displayed impaired curli formation yet was highly effective at gating the CsgG pore to confer erythromycin resistance. The F68S mutant also appeared more stable on Western blot. Thus, this mutation appears to be increasing the stability of the protein in a way that causes it to block CsgG and reduce the secretion of CsgA.

FUTURE DIRECTIONS

In order to consider the role these sites play in the interaction between CsgE and CsgA, future experiments could evaluate the ability of these CsgE mutants to inhibit CsgA polymerization. WT CsgE has been shown to inhibit CsgA polymerization at a near 1:1 molar ratio[19]. Since the W49S and W52S mutants displayed a Congo red binding phenotype comparable to wild type (Figure 3BC), this may indicate that these mutations still permit normal interaction with CsgA. If this is the case, these mutants should be able to inhibit CsgA polymerization at similar molar ratios to wild type. However, the W49S and W52S mutants were also susceptible to erythromycin in the erythromycin sensitivity assays (Figure 6AB). It is possible that since these mutants have impaired interactions with CsgG, the pore is more permissive, and the CsgE mutants do not need to interact with CsgA at all.

The F68S mutant displayed an intermediate Congo red binding phenotype (Figure 3D), and cells containing this mutation displayed less CsgA on Western Blot (Figure 4F). Thus, one possibility is that the mutation at this site hinders the ability of CsgE to interact with CsgA. If this mutant is unable to secrete CsgA normally, then perhaps it will not be able to inhibit CsgA polymerization. On the other hand, since the F68S mutant displayed increased rescue ability in the erythromycin sensitivity assay (Figure 6C), perhaps the decrease in CsgA secretion is a secondary effect of the F68S mutant's increased ability to block the CsgG pore. Measuring the ability of these mutants to inhibit CsgA polymerization would provide a better understanding of the role these sites play in interacting with CsgA.

CsgA polymerization can be measured using thioflavin T (ThT), a fluorescent dye that binds to amyloid fibers [15]. The assay would be performed by incubating purified CsgE mutants

with purified CsgA and measuring fluorescence over time with ThT. If the above predictions are correct, decreased fluorescence should be observed when CsgA is incubated with the W49S and W52S mutants, as they inhibit polymerization. If increased polymerization is observed with the F68S mutant, then the mutation at this site hinders the ability of CsgE to inhibit CsgA polymerization, suggesting that this site is important for interactions between the two proteins. However, if the F68S mutant also displays decreased fluorescence, then this mutation may not interfere with the ability of CsgE to inhibit CsgA polymerization, suggesting that perhaps the decrease in curli fiber formation is a result of the F68S mutant blocking the CsgG pore and preventing secretion of CsgA.

CONCLUSION

CsgE presents a promising area of future research into the mechanisms behind curli fiber secretion and formation. Three sites of interest have been identified that may play important roles in how CsgE interacts with other proteins involved in curli biogenesis. W49S and W52S mutations resulted in a Congo red binding phenotype comparable to wild type, demonstrating that mutations at these sites still permit CsgA secretion and curli fiber formation. However, the W49S and W52S mutations were unable to confer erythromycin resistance in erythromycin sensitivity assays. Thus, mutations at these sites interfere with the ability of CsgE to block or form a complex with the CsgG pore, suggesting that these sites are important for interactions between CsgE and CsgG. However, since these mutants were still able to form curli fibers, it may be the case that these mutants are unable to block the CsgG pore, keeping it open and allowing CsgA to diffuse across the outer membrane.

The third site assessed was at the 68th amino acid. A Phe-Ser mutation here resulted in impaired curli fiber formation, as seen through an intermediate pink phenotype on Congo red. Evaluation of CsgE levels via Western blot also revealed that the F68S mutant was present in greater quantities, indicating that this mutation may be making CsgE more stable. When the erythromycin sensitivity assay was performed, the F68S mutant conferred as much or even greater erythromycin resistance than WT CsgE. Thus, the mutation at this site may be changing the stability of the protein in a way that enhances its ability to block the CsgG pore while not allowing CsgA to be secreted normally.

While more research is necessary to gain a better understanding of the gating mechanism that occurs between CsgE and CsgG, it appears that all three sites play a role in this

interaction, particularly residues 49 and 52. Assays for CsgA polymerization could also be performed to evaluate how these mutants interact with CsgA. An understanding of how these sites affect the function of CsgE allows for a better understanding of the steps and regulations involved in curli secretion and formation, and thus potential targets for their inhibition.

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