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Secretion of curli fibre subunits is mediated by the outer membrane-localized CsgG protein

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Summary

Produced by many *Enterobacteriaceae* spp., curli are biologically important amyloid fibres that have been associated with biofilm formation, host cell adhesion and invasion, and immune system activation. CsgA is the major fibre subunit and CsgE, CsgF and CsgG are non-structural proteins involved in curli biogenesis. We have characterized the role of CsgG in curli subunit secretion across the outer membrane. Directed mutagenesis of CsgG confirmed that its activity is dependent on localization to the outer membrane. Rotary Shadow electron microscopy of purified CsgG suggested that this protein assembles into an oligomeric complex with an apparent central pore. Oligomeric CsgG complexes were confirmed using co-purification experiments. Antibiotic sensitivity assays demonstrated that overexpression of CsgG rendered *Escherichia coli* susceptible to the antibiotic erythromycin. A 22-amino-acid sequence at the N-terminus of CsgA was sufficient to direct heterologous proteins to the CsgG secretion apparatus. Finally, we determined that CsgG participates in an outer membrane complex with two other curli assembly proteins, CsgE and CsgF.

Introduction

Bacteria use a variety of extracellular fibres to mediate interactions with other cells and with their environment. Assembly of these fibres is complex and often includes chaperon proteins and outer membrane usher-like proteins that are dedicated to the secretion and proper localization of the fibre subunit proteins. Curli represent a class of thin (6–8 nm), highly aggregated surface fibres that are part of a complex extracellular matrix promoting biofilm and other community behaviours in *Escherichia coli* (Zogaj *et al.*, 2001; 2003). Curli also confer binding to fibronectin, laminin, plasminogen and human contact phase proteins (Olsen *et al.*, 1989; Sjobring *et al.*, 1994; Ben Nasr *et al.*, 1996). Among bacterially produced fibres, curli are distinguished by their unusual resistance to chemical and thermal denaturation and by their ability to bind the dyes Congo red (CR) and thioflavin T (ThT). These are properties shared by a growing number of eukaryotic fibres collectively know as amyloids. Amyloid fibres, or the process of amyloid formation, are proposed to cause cell and tissue damage associated with many neurodegenerative diseases (Lashuel *et al.*, 2002; Kayed *et al.*, 2003).

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Curliated bacteria stain red when grown on plates supplemented with CR, which provides a convenient way to identify genes important for curli production (Collinson *et al.*, 1993). CR binding has been observed in *Salmonella enterica*, *Klebsiella* spp., and *Escherichia* spp., and the genes necessary for curli production have been found in numerous clinically important *Enterobacteriaceae* that form biofilms (Zogaj *et al.*, 2003). Curli assembly requires the co-ordinated effort of proteins encoded by the *csgBA* and *csgDEFG* operons. The *csgBA* operon encodes two homologous proteins (CsgA and CsgB) that are secreted into the extracellular environment (Hammar *et al.*, 1995; 1996). At the cell surface, CsgA is assembled into a stable CR-binding amyloid fibre in the presence of CsgB. In the absence of the CsgB nucleator, CsgA is secreted from the cell in a soluble, unassembled state. This soluble CsgA can polymerize into curli fibres if it contacts an adjacent cell expressing the CsgB nucleator (and not expressing CsgA) by interbacterial complementation (Hammar *et al.*, 1996; Bian and Normark, 1997).

The *csgDEFG* operon encodes CsgD, a transcriptional activator of curli synthesis, and three putative assembly factors, CsgE, CsgF and CsgG (Hammar *et al.*, 1995; Romling *et al.*, 1998a). Efficient curli assembly requires the CsgE, CsgF and CsgG proteins. CsgG is a lipoprotein localized to the periplasmic side of the outer membrane (Loferer *et al.*, 1997). In the absence of CsgG, CsgA and CsgB are unstable and curli assembly does not occur. At least three models exist to explain these observations. The first suggests that CsgG stabilizes curli subunits in the periplasm, allowing them to be secreted by an unidentified secretion apparatus. The second posits that CsgG assembles into a pore that shuttles curli subunits across the outer membrane. A final model suggests that CsgG stabilizes the subunits in the periplasm and is directly involved in their secretion. The last two models assume that curli subunit stability is coupled to their secretion across the outer membrane.

Secretins are a well-characterized class of bacterial outer membrane proteins thought to function as secretion pores. Secretins often require accessory lipoproteins called pilot proteins for their oligomerization and/or outer membrane localization (Hardie *et al.*, 1996). Lipoproteins have also been implicated directly in the secretion step – perhaps as the secretion pore (Schmidt *et al.*, 2001; Bose and Taylor, 2005). Interestingly, while secretins have marked sequence similarity within their C-terminal domains, the lipoproteins hypothesized to function as secretion pores share no apparent sequence similarity (Brok *et al.*, 1999; Schmidt *et al.*, 2001; Bose and Taylor, 2005). In this study, we have characterized the lipoprotein CsgG and our results are consistent with the model that CsgG forms an outer membrane channel that stabilizes the curli subunit proteins by mediating their translocation across the outer membrane. We also demonstrated that CsgG interacted with two periplasmic proteins, CsgE and CsgF, required for efficient curli assembly *in vivo*. Collectively, our data suggest a new model of curli biogenesis where CsgG is the point of convergence in a pathway enabling curlin subunit secretion.

Results

The role of CsgG in curlin secretion was investigated. CsgG is an outer membrane-localized lipoprotein required for curli production and for CsgA and CsgB stability (Loferer *et al.*, 1997). A convenient measure of subunit secretion is the ability of a cell to act as a CsgA donor or acceptor during interbacterial complementation (Hammar *et al.*, 1995; Chapman *et al.*, 2002). For example, a *csgB*$^-$ or *csgF*$^-$ strain will secrete soluble CsgA to the cell surface that can be polymerized on the surface of a CsgB$^+$ acceptor strain. As a *csgG*$^-$ strain does not assemble curli, interbacterial complementation was used to assess its ability to produce functional CsgA and CsgB proteins. A *csgG*$^-$ strain (LSR1) is white when streaked on CR plates. As shown in Fig. 1A, a *csgG*$^-$ strain was unable to accept CsgA from a *csgB*$^-$ (MHR261) donor, nor was it able to donate CsgA to a *csgA*$^-$ (MHR204) acceptor strain.

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To assess whether CsgG was sufficient for CsgA secretion, CsgA was expressed from a plasmid in the complete Δ*csg* strain, LSR12. In this system, the expression of the Csg proteins is under the control of IPTG. As shown in Fig. 1B, accumulation of CsgA in the supernatant was observed in strains that expressed CsgG (Fig. 1B, lanes 2, 3 and 5). Expression of CsgE did not significantly alter CsgA accumulation or secretion under these conditions. Strains expressing CsgE, CsgF and CsgG from a single operon contained on plasmid pMC5 consistently secreted less CsgA than strains expressing CsgG from pMC1 (Fig. 1B) (Chapman *et al.*, 2002). Notably, CsgE is not required for CsgA secretion when CsgG is overexpressed from the IPTG-inducible *trc* promoter (Fig. 1B), but CsgE is critical for CsgA stability and curli formation when CsgG is expressed at wild-type (WT) levels from the chromosome (Chapman *et al.*, 2002).

The observation that coexpression of CsgA and CsgG is sufficient for CsgA secretion suggests that these proteins interact. CsgA can be divided into at least three identifiable domains: the N-terminal Sec-dependent signal sequence, the first 22 amino acids of the mature protein, and a C-terminal domain that is predicted to form the amyloid core of the fibre (Collinson *et al.*, 1999). The N-terminal 22 amino acids of the mature CsgA protein do not constitute an integral part of the fibre (Collinson *et al.*, 1999), but are required for CsgA stability and, possibly, secretion (L. Robinson, unpubl. results). To assess whether the mature N-terminal 22 amino acids of CsgA are sufficient for interaction with CsgG, the N-terminal 42 amino acids of premature CsgA (including the CsgA Sec-dependent signal sequence and the first 22 amino acids of the mature protein) were fused to the mature PhoA protein (Fig. 1C). This fusion protein was expressed from the *araBAD* promoter in plasmid pAph2. A second PhoA construct was made that lacked the 22 N-terminal residues of CsgA, called pAph1 (Fig. 1C). A C-terminal HA epitope tag on PhoA allowed for the immunoprecipitation of this protein using α-HA antibodies. Six C-terminal histidine residues were added to CsgG and this fusion construct was cloned behind the *trc* promoter in pTRC99A creating pMC2. This plasmid was able to complement CR-binding and curli formation to a *csgG* null mutant (M.R. Chapman, unpubl. data). This construct also drove CsgG expression in cells growing logarithmically in Luria–Bertani (LB) media – a condition where expression from *csgBA* and *csgDEFG* promoters is undetectable (Romling *et al.*, 1998b). Loferer *et al.* (1997) reported that when CsgG was expressed from its native promoter it fractionated exclusively to the Triton-X100 insoluble outer membrane fraction. We have confirmed these results and we have also determined that, when total membranes are treated with sarkosyl, natively expressed CsgG is found almost entirely in the sarkosyl insoluble outer membrane fraction (data not shown). When CsgG is overexpressed from the *trc* promoter, significant amounts are found in both sarkosyl soluble and insoluble fractions (Fig. 1D, lanes 4–7). The sarkosyl soluble protein observed when CsgG is overexpressed may represent CsgG that is associated with the inner membrane or CsgG that is only weakly interacting with the outer membrane at the time of fractionation. LSR12 (C600::Δ*csg*) containing pMC2 was transformed with either pAph1 or pAph2. We found that CsgG was present in the Elugent soluble material derived from the sarkosyl insoluble fraction when CsgG was expressed with either WT PhoA (Aph1) or with the CsgA–PhoA fusion protein (Aph2) (Fig. 1D, lanes 6 and 7). CsgG was specifically immunoprecipitated with α-HA antibodies only when CsgG and the CsgA-PhoA-HA fusion protein (Aph2) were coexpressed (Fig. 1D, lane 9). CsgG was not immunoprecipitated when coexpressed with WT PhoA-HA (Fig. 1D, lanes 8), suggesting that the 22 N-terminal amino acids of CsgA are sufficient for mediating an interaction between CsgG and CsgA.

Purification and structural analysis of CsgG

CsgG-his was expressed from pMC2 and purified using affinity chromatography as described in the *Experimental procedures*. Outer membranes were recovered by detergent extraction and Elugent soluble material was passed over a nickel NTA column. Nickel NTA-purified CsgG

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migrated near its predicted molecular weight of 29 kDa (Fig. 2A), although a smaller band was consistently observed in elution fractions that contained full-length CsgG (Fig. 2A, lane 6). The lower molecular weight band is apparently N-terminally truncated CsgG, as this band was recognized by α-His antibodies (data not shown).

Gram negative outer membrane secretion pores have been resolved by electron microscopy (EM) as 12–20 nm wide barrel-like structures (Thanassi *et al.*, 1998; Brok *et al.*, 1999). Observation of purified CsgG-his by rotary shadowing EM revealed discrete structures of approximately uniform shape and size. The observed structures were 12–15 nm wide with an apparent central pore of approximately 2 nm (Fig. 2C). The structures observed in Fig. 2C suggest that CsgG forms pore-containing oligomers in the outer membrane. Although these images suggest that CsgG forms an oligomeric structure, rotary replication can result in an enlargement of the imaged objects, which complicates precise estimation of the size of the oligomer complex or pore size (Thanassi *et al.*, 1998). Therefore, we utilized two differently tagged versions of CsgG to biochemically confirm CsgG-CsgG interactions. Strain C600 containing inducible plasmids that express CsgG-his (pMC2) and CsgG-HA (pLR92) was grown to mid-log phase and induced as described in the *Experimental procedures*. Outer membrane fractions were collected from strains containing pMC2 alone, pLR92 alone, or pMC2 and pLR92 together. The Elugent soluble material derived from these outer membrane fractions was loaded onto a Ni-NTA column and CsgG-his was immobilized on the column. Proteins were eluted from the column and the eluates were probed with anti-HA antibodies. CsgG-HA was detected in the eluate only when CsgG-his was coexpressed, suggesting that CsgG-his and CsgG-HA formed a complex that was stable throughout Ni-NTA purification (Fig. 2B).

Antibiotic sensitivity assays

We next ascertained whether CsgG expression modified the permeability of the outer membrane by using antibiotic sensitivity assays. Erythromycin does not normally pass the Gram negative outer membrane because of hydrophobic repulsion forces. Therefore, this bacterio-static antibiotic can be used to detect the presence of channels in the outer membrane (Augustus *et al.*, 2004). As shown in Fig 3A, 30 μg ml^{-1} erythromycin did not appreciably affect growth of LSR12 containing vector alone. In contrast, LSR12 containing pMC1 displayed severe growth defects in the presence of erythromycin (Fig. 3A). Therefore, it appeared that CsgG expression increased outer membrane permeability, thus allowing erythromycin to enter the cell and poison translation. This is in agreement with our observation that CsgG assembled into pore-like structures with an apparent central cavity of 2 nm, which would be large enough to allow erythromycin to pass. Importantly, bacteria expressing CsgG did not exhibit growth defects in the absence of erythromycin (Fig. 3A), suggesting that CsgG did not grossly affect membrane integrity. Erythromycin sensitivity was also observed when MC4100 cells overexpressing CsgG were grown on YESCA plates (data not shown). Vancomycin is another antibiotic that is often used to gauge membrane permeability because, like erythromycin, it cannot pass the hydrophobic outer membrane (Schmidt *et al.*, 2001). We observed relatively modest growth inhibition using vancomycin, even in cells overexpressing CsgG from pMC1 (Fig. 3B), suggesting that CsgG-dependent erythromycin sensitivity is not due to a general membrane defect and may be due to presence of a CsgG pore in the outer membrane. At twice the size of erythromycin (1440 Da versus 740 Da for erythromycin), we reasoned that vancomycin might not pass through the pore fashioned by CsgG oligomers, although it is possible that chemical differences between the antibiotics are responsible for the exclusion of vancomycin from CsgG-expressing cells.

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Lipid modification is required for outer membrane localization

CsgG has been demonstrated to be a lipoprotein localized to the outer membrane. Consequently, it has been suggested that CsgG is targeted to the outer membrane by the LOL transport system (Loferer *et al.*, 1997). Bacterial lipoproteins are modified on a cysteine residue located at the N-terminus (Narita *et al.*, 2004). CsgG contains a conserved N-terminal cysteine residue that is the putative site of lipidation. We constructed a substitution mutant of this cysteine residue (C16A) and found its expression to be toxic to cells (our unpublished data). Therefore, we removed the CsgG lipoprotein signal sequence, including the N-terminal cysteine, and replaced it with the signal sequence from the PhoA protein (Fig. 4A). The expression of this protein chimera, called CsgGss, was induced in log phase cells and then localized using detergent fractionation. WT CsgG consistently localized with outer membrane fractions, while CsgGss localized predominately with the soluble periplasmic fraction (Fig. 4B). The ability of CsgGss to complement a *csgG*$^-$ mutant was tested. No CR binding was detected in a *csgG*$^-$ mutant, although this strain could be complemented by expression of WT *csgG* from the *trc* promoter in pMC1 (Fig. 4C). Partial complementation was achieved when WT *csgG* was expressed from the *csgBA* promoter in pLR93. In contrast, CsgGss was unable to complement the *csgG*$^-$ mutant when expressed from either the *trc* or *csgBA* promoters (Fig. 4C). Interestingly, the expression of CsgGss abolished the CR positive phenotype of an otherwise WT strain (Fig. 4C), suggesting that CsgGss is able to interfere with WT CsgG function.

Because the CsgA and CsgB proteins are unstable without CsgG, we tested the ability of CsgGss to stabilize the curli subunits in the absence of WT CsgG. As shown in Fig. 4D, CsgA and CsgB do not accumulate to WT levels when CsgGss is expressed in a *csgG*$^-$ strain, suggesting that proper CsgG localization to the outer membrane is required for the chaperone-like activity of CsgG (Fig. 4D).

Complex with other Csg proteins

CsgG is expressed from an operon along with three other proteins that are known to play a role in curli formation. CsgD is a transcriptional activator required for curli gene expression, while CsgE and CsgF are chaperone-like proteins that facilitate curli formation (Chapman *et al.*, 2002; Chirwa and Herrington, 2003; Gerstel *et al.*, 2003). However, the roles of CsgE and CsgF in curli formation are poorly understood. We tested whether CsgE or CsgF could interact with CsgG at the outer membrane. To facilitate this analysis, the CsgE and CsgF proteins were epitope tagged with AU1 or HA respectively. These fusion proteins were able to complement CR binding to *csgE* and *csgF* chromosomal deletion strains (data not shown). C600 with pMC2 and pBAD33 or pLR58 (*csgF-HA* in pBAD33) was grown and the expression of CsgF-HA and CsgG-his was induced with arabinose and IPTG. Outer membranes were prepared after 1 h of induction and CsgF-HA was immunoprecipitated with α-HA antibodies. CsgG was detected in the Elugent soluble outer membrane fraction with or without coexpression of CsgF-HA (Fig. 5A, lanes 4 and 5), but was specifically co-immunoprecipitated with α-HA antibodies only when CsgF-HA was present. This demonstrated that CsgF and CsgG physically interacted at the outer membrane. C600 containing pMC2 and pBAD33 or pLR169 (*csgE-AU1* in pBAD33) was grown to induce CsgG-his and CsgE-AU1 expression. Proteins contained within the sarkosyl soluble inner membrane and Elugent soluble outer membrane fractions were immunoprecipitated with α-AU1 antibodies. As shown in Fig. 5B, CsgE-AU1 and CsgG-his were co-immunoprecipitated with α-AU1 antibodies from the Elugent soluble fractions. Immunoprecipitation of CsgG-his was completely dependent on expression of CsgE-AU1 (Fig. 5B). CsgG-his was not co-immunoprecipitated with CsgE-AU1 when sarkosyl soluble fractions were used (Fig. 5B, lanes 6–7 and 10–11). These data suggest that CsgG is an integral part of an outer membrane secretion complex that contains at least two other curli assembly proteins, CsgE and CsgF.

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Discussion

Curli biogenesis is a complex process that requires several proteins, including those encoded by the *csg* operons. The lipoprotein CsgG forms an oligomeric structure in the outer membrane that is required for the secretion of the CsgA and CsgB proteins to the cell surface. The localization of CsgG to the outer membrane is dependent on posttranslational acylation of the cysteine located at the first residue of the mature protein. Overexpression of CsgG renders logarithmically growing cells susceptible to the hydrophobic antibiotic erythromycin, most likely by a specific change in the permeability of the outer membrane. CsgA secretion and stability is dependent on the N-terminal 22 amino acids of CsgA. These 22 amino acids can also direct non-curli proteins to form a complex with CsgG. CsgE and CsgF also participate in a complex with CsgG, perhaps modifying its secretion activity.

Bacteria use a variety of mechanisms to shuttle proteins to the cell surface, and lipoproteins are often key components of these systems. Lipoproteins that function in protein secretion fall into one of two functionally distinct classes. The first class represents lipoproteins that chaperone secretin proteins to the outer membrane. Pugsley and colleagues have coined the term ‘pilot’ protein to describe such proteins. The second class of lipoproteins involved in protein secretion is postulated to form an outer membrane pore through which substrate proteins are channelled. Members of several terminal secretion systems predicted to be lipoproteins are directly involved in protein secretion across the outer membrane, including BfpB (Ramer *et al.*, 1996; Schmidt *et al.*, 2001), CfcD (Mundy *et al.*, 2003) and TcpC (Bose and Taylor, 2005). CsgG does not share significant sequence similarity to any of the lipoproteins known to participate in bacterial secretion, yet CsgG homologues are present in many Gram negative bacteria – including many clinically important members of *Enterobacteriaceae* (Zogaj *et al.*, 2003 and our unpublished data).

In the absence of CsgG, the CsgA and CsgB proteins are not secreted to the cell surface, yet they do not accumulate in the periplasmic space (Loferer *et al.*, 1997). Currently, there are at least two models that explain why CsgA and CsgB are unstable in the absence of CsgG. CsgG might act as a chaperone protein, stabilizing CsgA and CsgB in the periplasm until they are secreted to the cell surface. Alternatively, CsgG may simply transport CsgA and CsgB to the cell surface where they are not subject to periplasmic proteases. To clarify the mechanism of CsgG, we asked if CsgG localization to the outer membrane was required for CsgA and CsgB stability. CsgG is modified with a palmitate group after secretion across the inner membrane (Loferer *et al.*, 1997). Therefore, the lipidation-specific signal sequence on CsgG was replaced with a general SEC-dependent signal sequence. This protein, called CsgGss, is not lipidated and localizes to the periplasm instead of the outer membrane. Furthermore, CsgGss is unable to restore curli production in a *csgG*$^{-}$ strain (Fig. 4C) and it does not act to stabilize the CsgA or CsgB proteins (Fig. 4D). The failure of CsgGss to stabilize curli subunits cannot be explained by an inability to bind subunits, as CsgGss and the CsgA–PhoA fusion specifically interacted in Far-Western assays (L. Robinson, unpubl. data). This suggests that CsgG must localize to the outer membrane in order to stabilize CsgA and CsgB and direct their secretion across the outer membrane. Therefore, stability and secretion of CsgA and CsgB appear to be tightly coupled. CsgA and CsgB may be inherently unstable in the periplasm and CsgG-mediated secretion helps to stabilize them by exporting them to the more forgiving extracellular space. The extracellular space is also where curli subunits are proposed to transition into the amyloid state, which might also lead to increased subunit stability (Hammar *et al.*, 1996).

The molecular mechanism behind the dominant negative phenotype observed when CsgGss is expressed along with WT CsgG is unclear. At least two possibilities exist to explain this result. As CsgG forms oligomeric structures in the outer membrane, it is possible that CsgGss can form a non-functional complex with WT CsgG, thereby preventing WT CsgG activity. As

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shown in Fig. 2, CsgG forms oligomeric structures and it is possible that CsgGss participates in this complex. Alternatively, expression of CsgGss may change the cellular environment such that curli formation is inhibited. For example, if CsgGss is not completely folded then inducible cell stress systems may be activated that negatively affect curli formation. One such system is the Cpx system that can be induced by misfolded proteins in the periplasm (Hung *et al.*, 2001;Nevesinjac and Raivio, 2005;Ruiz and Silhavy, 2005). Not only does the Cpx system upregulate the expression of proteins with protease activity, which might degrade WT CsgG or the curli subunits, but CpxR has also been shown to downregulate the *csg* operons at the transcriptional level (Prigent-Combaret *et al.*, 2001).

CsgA that has been secreted by CsgG-expressing cells is found in an unstructured state upon purification (Chapman *et al.*, 2002). The size of the structures observed in Fig. 2C is in agreement with the idea that the curli subunits may be at least partly unfolded during translocation across the outer membrane. However, the mechanism by which CsgA could be maintained in this unfolded state is unclear. CsgG probably does not work alone during curli assembly, despite the discovery that overexpression of CsgG in logarithmically growing cells results in CsgA secretion (Fig. 1B), In this experiment, CsgG and CsgA were expressed from inducible promoters and CsgA was almost exclusively found in the extracellular space. Neither CsgE nor CsgF augmented CsgA secretion when CsgG was overexpressed. Yet, CsgE is required for CsgA and CsgB secretion when cells are grown on YESCA plates and the curli proteins are expressed from their native promoters (Chapman *et al.*, 2002). Because CsgE and CsgF interact with CsgG at the outer membrane, it is possible that under native conditions CsgE and CsgF increase the specificity or efficiency of CsgG-mediated secretion. Delineating the molecular details of CsgE and CsgF function during curli formation will help clarify this unique secretion system.

Experimental procedures

Plasmids, strains, growth conditions and antibodies

Expression of *csg* genes at WT levels from MC4100 chromosome or pLR1-derived plasmids was accomplished by growing cells on YESCA agar [10 g Casamino acids, 1 g yeast extract, 20 g agar (Fisher, Fairlawn, NJ) in 1 l] at 26°C for 48 h. YESCA agar plates supplemented with 10 μg ml^{-1} CR (Sigma, St. Louis, MO) were used to monitor curli production on colonies. Expression of genes cloned behind the *trc* promoter in pTRC99A or the *ara* promoter in pBAD33 was induced by addition of 0.4 mM IPTG or 0.4% (w/v) arabinose respectively. Antibiotics were added at the following concentrations as needed: kanomycin 50 μg ml^{-1}, ampicillin 100 μg ml^{-1} and chloramphenicol 20 μg ml^{-1}. The *csgBA* promoter was amplified from MC4100 as a BglII/PstI fragment using primers LR1F and LR1R (Table S1) and cloned into the BamHI/PstI sites of pACYC177 to create pLR1. An NcoI site incorporated into the reverse primer LR1R allowed cloning of the NcoI/PstI fragment of pLomp4 (Table 1) into pLR1, which added BamHI and KpnI sites and created pLR2. Western blots were probed as indicated in the *Results* with the following antibodies: polyclonal antiserum raised against Ni-NTA purified CsgG-his in rabbits by Proteintech Group, Chicago, IL, rabbit polyclonal antibody raised against the CsgB peptide EGSSNRAKIDQTGDY (Sigma, St. Louis, MO), rabbit polyclonal antibody raised against CsgA (Hammar *et al.*, 1996), or commercially available antibodies against commonly used epitopes, as indicated when used.

Purification of CsgG

CsgG was purified under non-denaturing conditions from strain C600 containing pMC2 and/or pLR92 as indicated in the *Results*. The strains were grown with aeration in LB broth containing 100 μg ml^{-1} ampicillin and/or 50 μg ml^{-1} kanomycin as appropriate. At OD_{600} of 0.6–1.0, *csgG-his* or *csgG-HA* expression was induced with 0.5 mM IPTG and/or 0.04%

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arabinose for 2 h. Bacteria were harvested and resuspended in 20 mM Tris.HCl (pH 8.0), lysed with a French press, and outer membrane collected by differential extraction with Sarkosyl (Thanassi and Hultgren, 2000). CsgG was extracted from the sarkosyl insoluble outer membrane fraction by treatment with 0.5% Elugent (Calbiochem, San Diego, CA). The Elugent soluble fraction was applied to a Ni-NTA column (Qiagen, Chatsworth, CA) in five column volumes of HNE (20 mM Hepes/300 mM NaCl/0.5% Elugent). The column was washed with five column volumes of HNE + 10 mM imidazole. An additional wash of five column volumes of HNE + 20 mM imidizole was performed for the co-purification experiment presented in Fig. 2B. CsgG bound to the column was eluted with HNE containing 100 mM imidazole.

Electron microscopy

CsgG was purified for inspection by EM analysis as described above with the following modifications: CsgG was extracted from the sarkosyl insoluble outer membrane fraction by treatment with 0.1% of the non-ionic detergent n-dodecyl-beta-D-maltoside (DDM) and the mobile phase of the Ni-NTA column was buffer A (20 mM Tris.HCl pH 8.0/150 mM NaCl/ 0.1% DDM). Purified CsgG was observed by rotary replication as described by Thanassi *et al.* (1998). Briefly, CsgG purified by Ni-NTA chromatography was adsorbed to mica chips before quick-freezing. These frozen samples were fractured and deep-etched by exposure to a vacuum. Replicas were constructed by rotary shadowing with platinum.

Cell fractionation to determine CsgG and CsgGss localization

C600 containing pMC1 or pLR16 was grown with aeration in LB broth and addition of 0.5 mM IPTG to the growth media induced *csgG* or *csgGss* expression. Cells were harvested and 0.5 g cell weight divided into two equal portions and either periplasm or membranes were isolated. The periplasmic fraction was obtained by suspending the cells in 20 mM Tris.HCl pH 8.0/20% sucrose/0.5 mM EDTA and 40 µl of 25× Protease Inhibitor Tablets (Roche, Indianapolis, IN) was added before treatment with 75 µg ml^{-1} lysozyme. This suspension was incubated on ice for 40 min and the reaction was quenched by addition of 1 M $MgCl_2$. Spheroplasts were pelleted by centrifugation at 10 000 *g* for 20 min and the supernatant retained for analysis. Membrane fractions were obtained using a modified protocol originally described by Loferer *et al.* (1997). Briefly, one half of the cells grown as described above were suspended in 20 mM Tris.HCl pH 8.0 and lysed with a French press before separating the periplasm and cytosol from the total membrane fraction by ultracentrifugation at 100 000 *g* for 1 h. The inner membrane was then solubilized in 0.5% sarkosyl and the sarkosyl insoluble outer membrane fraction pelleted by centrifugation at 100 000 *g* for 1 h. The pellet was suspended in HNE and insoluble material pelleted by centrifugation at 100 000 *g* for 30 min. The Elugent soluble fraction was retained for analysis. The fractions obtained by these procedures were subjected to SDS-PAGE and the presence of CsgG or CsgGss was detected by immunoblotting with rabbit anti-CsgG antibodies.

Immunoprecipitation

Elugent soluble outer membrane material was prepared as described above from the strains described in the *Results* section. One millilitre of the preparation was combined with anti-HA or anti-AU antibody at the concentration recommended by the manufacturer (Covance, Denver, PA) before rocking 1 h at 4°C. Twenty-five microlitres of Protein A-agarose bead slurry (Sigma, St Louis, MO) was added and the mixture rocked 1 h at 4°C. The beads were pelleted and washed three times in HNE before resuspension in 50 µl 2× SDS loading buffer.

Antibiotic sensitivity assays

Plasmid pTRC99A (empty vector) or pMC1 (P*trc-csgG*) were transformed into LSR12 (MC4100::Δ*csgDEFG*; Δ*csgBA*). Strains were grown to stationary phase in LB, diluted 1:100

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in fresh media, and grown with agitation for 30 min. At this point, 0.05 mM IPTG was added to the cultures, and bacteria were grown another 30 min before addition of erythromycin or vancomycin. Antibiotic addition was designated as time zero and OD_{600} measured every 30 min for 300 min. The OD_{600} was measured using a Molecular Dynamics UV-Vis spectrophometer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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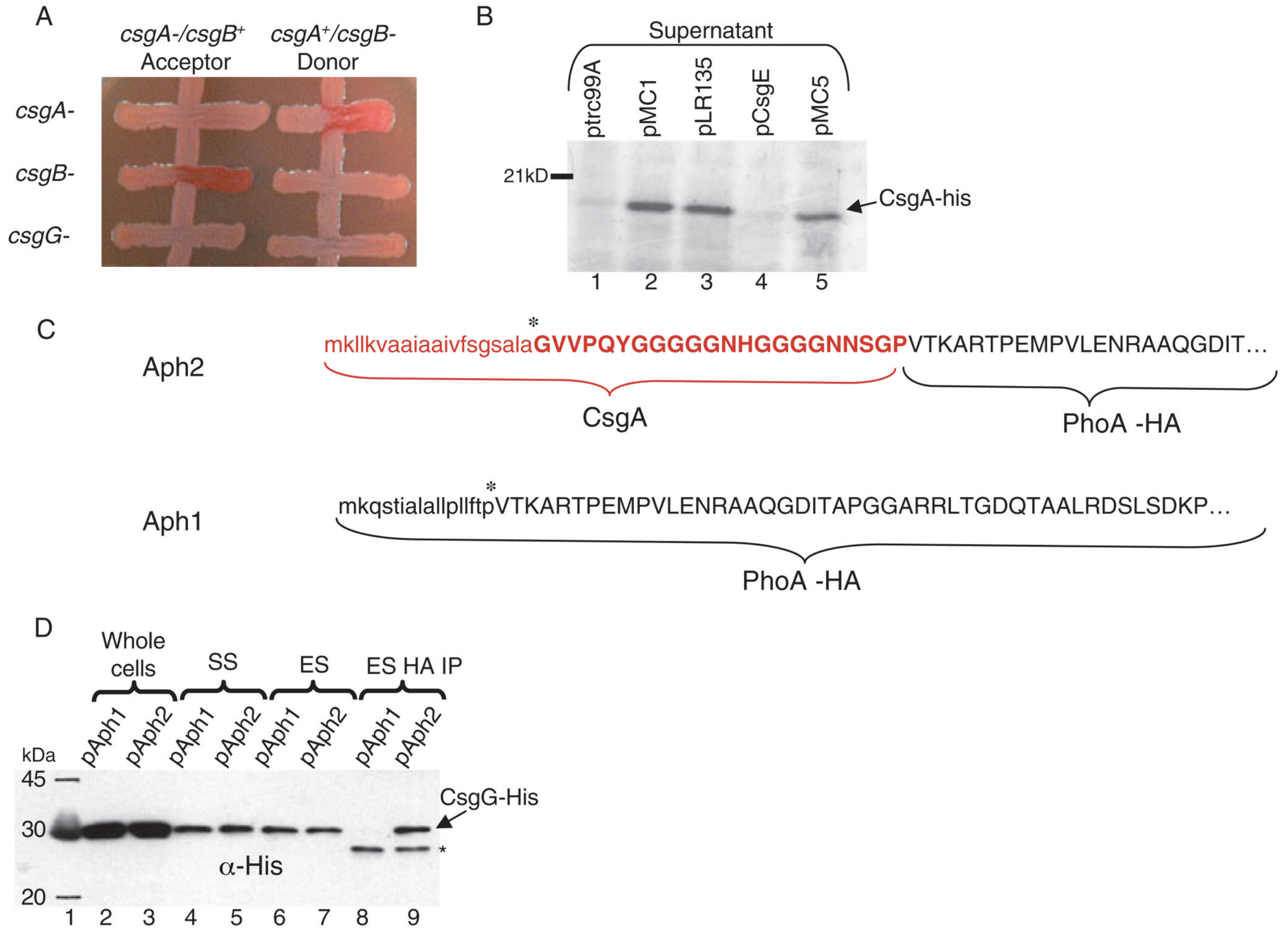

Fig. 1. CsgG is required for interbacterial complementation and CsgA secretion

A. Interbacterial complementation and CR binding of *csgG*$^{-}$ mutants. The *csgB*$^{+}$ acceptor strain MHR204 and the *csgA*$^{+}$ donor strain MHR261 were streaked from the top of the plate to the bottom. The horizontal cross-streaks were made from left to right with the indicated strains.

B. Expression and batch purification of CsgA-his. Supernatants from cells containing pMC3 (*csgA*$^{+}$) and the indicated plasmids were collected and mixed with 20 μl of Ni-NTA beads. After incubation for 20 min at room temperature with gentle rocking, samples were briefly centrifuged to pellet beads, decanted and resuspended in 1 × SDS loading buffer. CsgA-his migrated with an apparent molecular mass of approximately 17 kDa and is indicated with an arrow.

C. N-terminal sequences of WT PhoA (Aph1) and the CsgA–PhoA fusion protein (Aph2). An asterisk indicates the predicted signal peptidase II cleavage site.

D. Co-immunoprecipitation of Aph2 and CsgG. CsgG-his was detected in cell fractions prepared from cells expressing Aph2 (lanes 3, 5, 7 and 9) or Aph1 (lanes 2, 4, 6 and 8) as described in the *Experimental procedures* by Western blot with α-His antibodies. Samples that were immunoprecipitated (IP) from the Elugent soluble (ES) fraction with α-HA antibodies were loaded into lanes 8 and 9. The sarkosyl soluble (SS) fraction is also shown (lanes 4 and 5). CsgG-his migrates at approximately 30 kDa and the asterisk indicates the α-HA IgG protein recognized by the α-His antibody in the immunoprecipitation samples.

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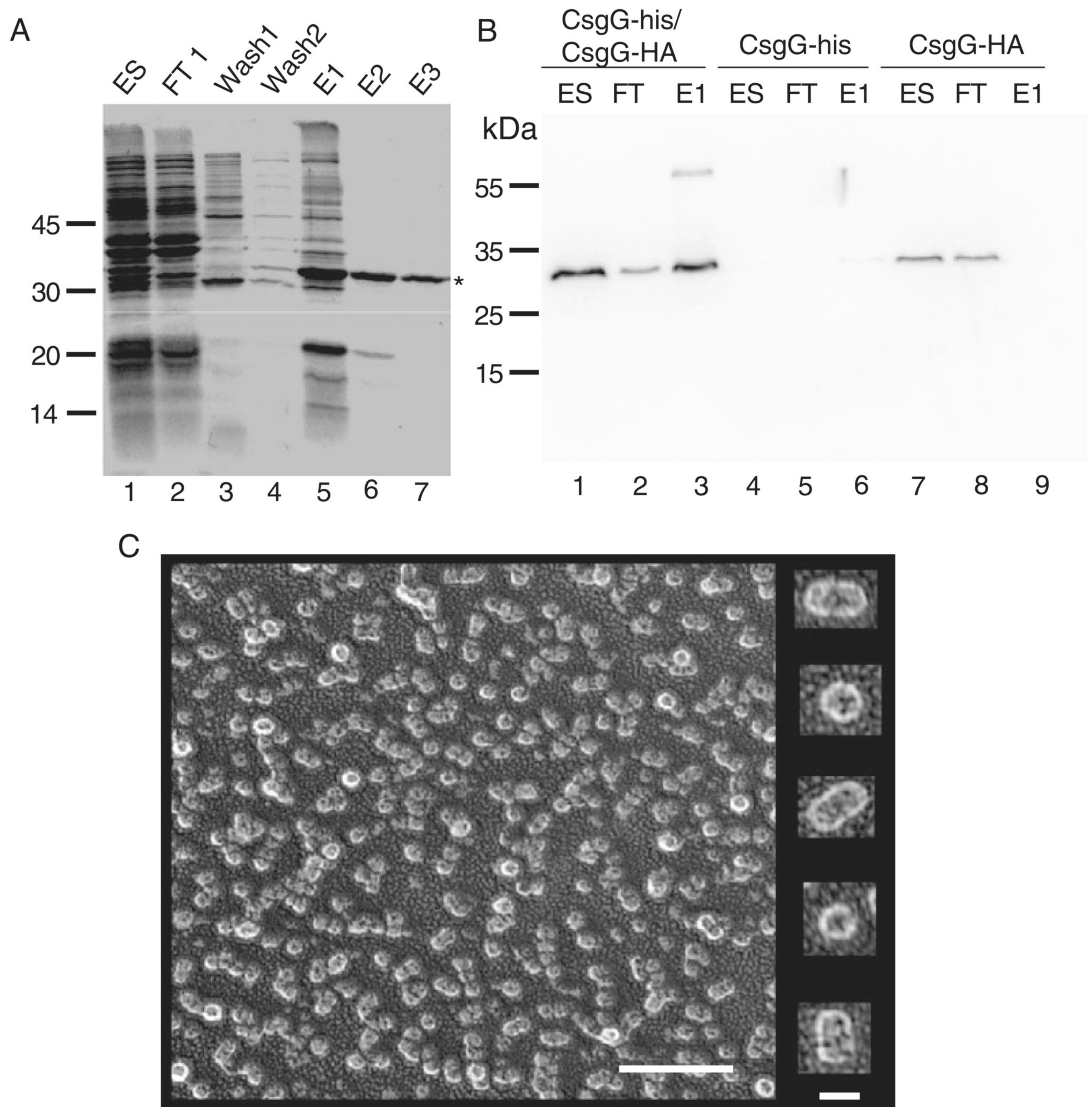

Fig. 2. Purification and structural analysis of CsgG

A. C600/pMC2 cells were harvested and the Elugent soluble (ES) outer membrane fraction (lane 1) was applied to a Ni-NTA column in five column volumes of HNE and 1 ml flow-through (FT) collected (lane 2). The column was then washed with five column volumes HNE containing 10 mM imidizole. The first (W1) and last (W2) millilitre of this wash was collected for analysis (lanes 3 and 4). CsgG-his bound to the column was eluted with HNE containing 100 mM imidizole and collected in three 1 ml fractions, E1–E3 (lanes 5–7). Fractions were resolved by SDS-PAGE and visualized by Coomassie brilliant blue staining. The asterisk at 30 kDa indicates the molecular weight of CsgG.

B. CsgG forms an oligomer in the outer membrane. ES outer membrane fraction from cells expressing CsgG-his (lane 4), CsgG-HA (lane 7), or both (lane 1) were isolated and CsgG-his was purified by Ni-NTA chromatography as described in *Experimental procedures*. Analysis of the fractions by Western blot analysis with α-HA antibodies revealed CsgG-HA in the eluate only when CsgG-his was coexpressed (lane 3). The band at ~55 kDa in lane 3 is an unidentified cross-reacting band that is sometimes seen when CsgG is purified from C600.
C. Rotary Shadow EM analysis of CsgG-his protein reveals donut-shaped structures approximately 13 nm in diameter. Scale bar left panel = 100 nm. Right panel shows enlargements of single particles from the left panel. Scale bar right panel = 15 nm. Rotary shadowing with platinum was performed as described in the *Experimental procedures*.

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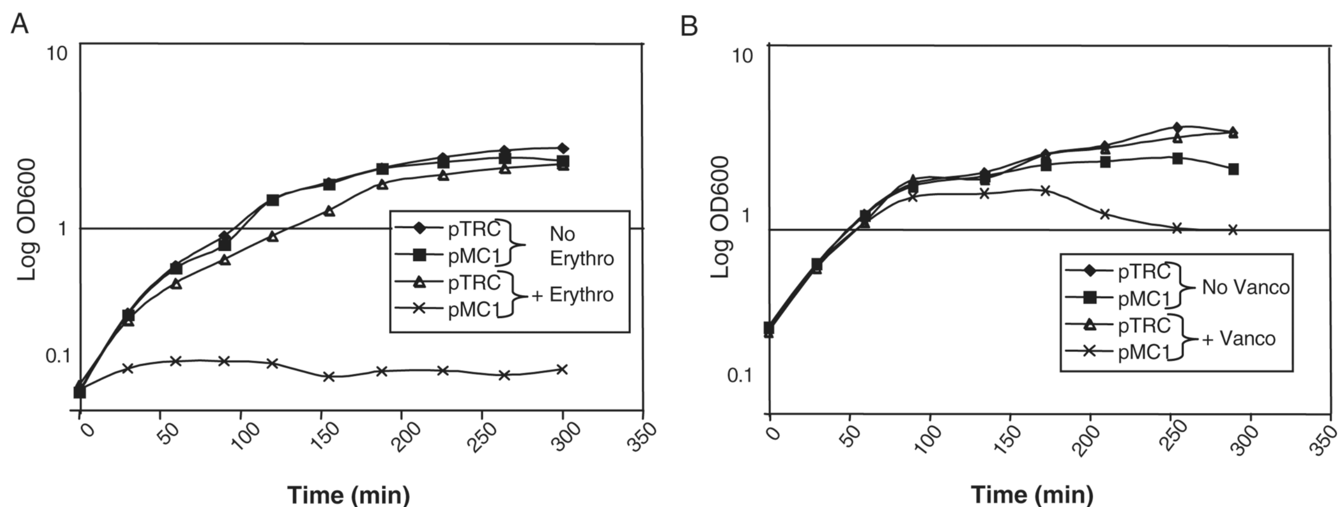

Fig. 3. Antibiotic sensitivity studies

A. Growth curve of *E. coli* strain C600 containing plasmid pTRC99A or pMC1 in the presence or absence of 30 μg ml^{-1} erythromycin. Strains were grown overnight, diluted 1:100 in LB and grown for 30 min in the presence of 0.05 mM IPTG, before erythromycin was added (time 0).

B. Growth curve of *E. coli* strain C600 containing plasmid pTRC99A or pMC1 in the presence or absence of 30 μg ml^{-1} vancomycin. Strains were grown overnight, diluted 1:100 in LB and grown for 30 min in the presence of 0.05 mg ml^{-1} IPTG, before vancomycin was added (time 0).

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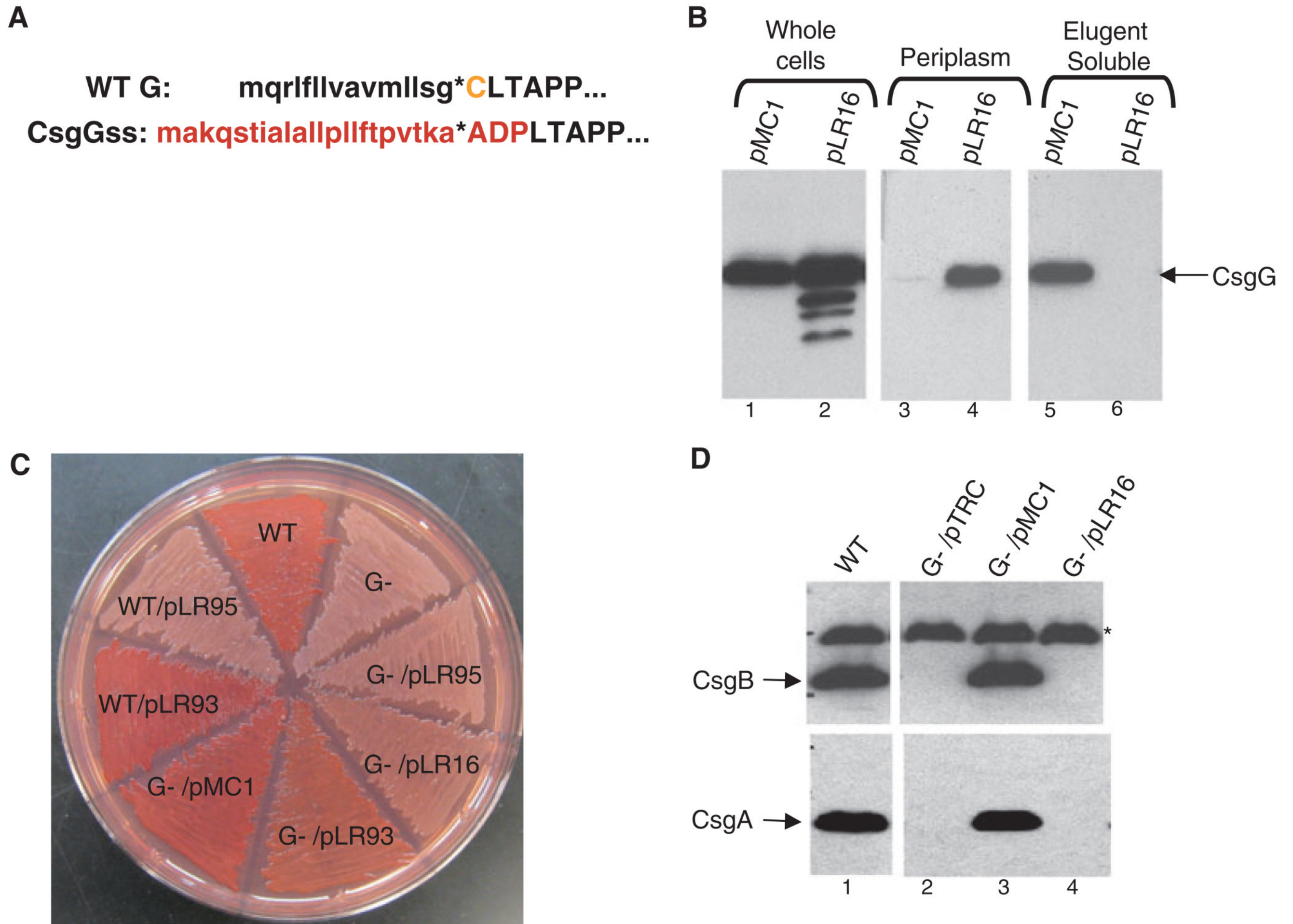

Fig. 4. Lipidation is required for CsgG activity

A. The N-terminal 21 amino acids of CsgG that encodes a SEC-dependent secretion sequence and lipoprotein modification site are shown. The asterisk indicates the putative signal peptidase II-dependent cleavage site. Shown in red are the N-terminal 25 amino acids from PhoA that were used to replace the lipoprotein signal sequence of CsgG.

B. Expression and localization of CsgG and CsgGss. Cells containing pMC2 or pLR16 were grown to mid-log phase and induced with 0.1 mM IPTG for 2 h before being harvested and fractionated as described in the *Experimental procedures*. CsgG and CsgGss were detected using α-His antibodies. Lanes 1 and 2 are whole cell lysates, lanes 3 and 4 are periplasmic fractions and lanes 5 and 6 are Elugent soluble outer membrane fractions. Equal amounts of CsgG-his and CsgGss-his fractions were loaded.

C. CR binding of *csgG*- strains containing the indicated plasmids after 48 h of growth at 26° C on YESCA plates.

D. Western analysis using α-CsgA or α-CsgB antibodies. Whole cell lysates of cells grown for 48 h on YESCA plates were treated with formic acid as described by Chapman *et al.* (2002) and probed with α-CsgA or α-CsgB antibodies. The asterisk indicates a non-specific protein recognized by the α-CsgB antibody.

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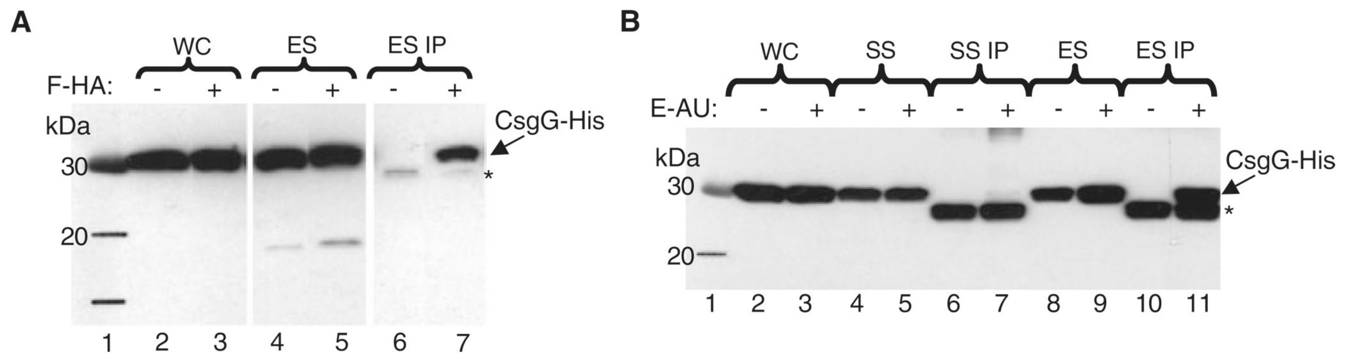

Fig. 5. CsgG interacts with CsgE and CsgF at the outer membrane

A. Cells expressing CsgG-his and CsgF-HA were fractionated and then immunoprecipitated with α-HA antibodies where indicated. Samples were blotted and probed with α-His antibodies to detect CsgG. CsgG migrates at approximately 30 kDa and is indicated with an arrow.

B. Cells expressing CsgG-his and CsgE-AU1 were fractionated prior to immunoprecipitation with α-AU antibodies. CsgG-his migrates at approximately 30 kDa and the asterisk indicates the IgG protein in the immunoprecipitation samples recognized by the α-His antibody. WC, whole cells; SS, sarkosyl soluble fraction; ES, Elugent soluble fraction; IP, immunoprecipitation.

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Table 1

Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source
C600	F- *thr leu thi lac tonA*	Campbell (1961)
LSR12	C600::Δ*csgDEFG*Δ*csgBA*	Chapman *et al.* (2002)
MC4100	F-*ara*D139 Δ(*argF-lac*)U169 *rsp*L150(*strR*) *rel*A1*flb*B5301 *deo*C1 *pts*F25 *rbs*R	Casadaban (1976)
LSR1	MC4100*csgG::Tn105*	This study
MHR204	MC4100*csgA::*Tn105	Hammar *et al.* (1996)
LSR10	MC4100::Δ*csgA*	Chapman *et al.* (2002)
MHR261	MC4100::Δ*csgB*	Hammar *et al.* (1996)
pTrc99A	Expression vector	Pharmacia Biotech
pMC1	*csgG* cloned into pTrc99A	Chapman *et al.* (2002)
pLR15	*csgG* amplified from pMC1 with Trc5F and LR15R cloned into pTrc99A	This study
pLR135	*csgE* amplified with LR135F and LR135R and cloned into KpnI/PstI sites of pLR15	This study
pLR16	DNA fragment encoding the PhoA signal sequence amplified with PhoSSF and LR16R1 cloned into the NcoI/BamHI sites of pTrc99A; sequence encoding mature CsgG with C-terminal 6 hi epitope amplified with LR16F2 and GhisR and cloned into BamHI/PstI sites of pTrc99A	This study
pCsgE	*csgE* amplified with PEF and PER and cloned into the NcoI/BamHI sites of pTrc99A	This study
pMC5	*csgEFG* cloned into pTrc99A	Chapman *et al.* (2002)
pMC2	*csgG-6his* amplified with Trc5F and GhisR and cloned into pTrc99A	This study
pLomp4	DNA fragment encoding the signal sequence of *E. coli* Lpp fused to residues 41–159 of OmpA cloned into the NcoI/BamHI sites of pTrc99A	This study
pBAD33	Expression vector	Guzman *et al.* (1995)
pLR29	*csgEFG* amplified from pMC5 using LR29F and LR29R and cloned into the KpnI/PstI sites of pBAD33.	This study
pLR46	DNA fragment encoding the N-terminus of PapD amplified with LR46F and LR46R cloned into the KpnI/Bgl2 sites of pLR29	This study
pLR60	CmR gene lacking an NcoI site amplified from pBAD33 with LR60F and LR60R and cloned into Msc1/Sca1 of pLR46	This study
pLR92	*csgG-HA* amplified from pMC1 with LR92F and LR92R and cloned into the SacI/PstI sites of pLR60	This study
pLR169	DNA fragment encoding N-terminal AU-tagged CsgE subcloned into NcoI/PstI sites of pLR92 from similarly digested pLR167	This study
pLR58	*csgF-HA* amplified with Trc5F and LR58R and cloned into the NcoI/PstI sites of pLR92	This study
pLR134	DNA fragment encoding the first 42 amino acids of CsgA, containing the s signal sequence and the first 22 residues of the mature protein, amplified with LR134F and LR134R cloned into the NcoI/PstI sites of pLR92	This study

Strain or plasmid	Relevant characteristics	Source
pAph2	DNA sequence encoding mature PhoA amended with a C-terminal HA tag amplified with Aph2F and PhoHAR cloned into the NcoI/PstI sites of pLR134. The resulting fusion encodes the first 42 amino acids of CsgA and the mature PhoA protein	This study
pAph1	*phoA-HA* amplified with Aph1F and PhoHAR and cloned into the SacI/PstI sites of pLR92	This study
pACYC177	Cloning vector	New England Biolabs
pLR1	*csgBA* promoter amplified with LR1F and LR1R and cloned into the BamHI/PstI sites of pACYC177.	This study
pLR2	The NcoI/PstI fragment of pLomp4 subcloned into pLR1	This study
pLR7	*csgGss-HA* amplified from pLR16 with PhoSSF and LR7R and cloned into the NcoI/PstI sites of pLR1.	This study
pLR167	DNA fragment encoding N-terminal AU-tagged CsgE amplified with LR169F and Trc3R and cloned into the BamHI/PstI sites of pLR7	This study
pLR95	The NcoI/PstI fragment of pLR16 containing the *csgGss* chimera subcloned into pLR1	This study
pLR93	The NcoI/PstI of pMC1 containing *csgG* subcloned into pLR1	This study
pMC3	*csgA-6his* cloned into pHL3	Chapman *et al.* (2002)

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