**Nucleator function of CsgB for the assembly of adhesive surface organelles in *Escherichia coli***

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Curli are surface organelles in *Escherichia coli* that assemble outside the bacterium through the precipitation of secreted soluble CsgA monomers, requiring the CsgB nucleator protein. Using immunoelectron microscopy and immunoblotting assays, CsgB is shown to be located on the bacterial surface and also as a minor component of wild-type curli. CsgB lacking its 20 N-terminal residues when fused to maltose-binding protein (MBP) can still trigger polymerization of CsgA monomers in *vivo*. However, the resulting surface organelles are only formed at one of the two bacterial poles and are morphologically distinct from wild-type curli. These Bfco organelles (CsgB-Free Curli-related Organelles) are highly regular structures reacting with anti-CsgA, but not anti-CsgB antibodies. The CsgB of the active MBP-CsgBII fusion is surface exposed but, unlike the native CsgB in wild-type curli, is not detectable in the Bfco organelles. Overexpression of csgB within a csgA mutant results in the formation of short CsgB polymers on the cell surface. It is suggested that in wild-type bacteria, both CsgA and CsgB are secreted proteins. Interaction between CsgA and CsgB triggers wild-type curli formation, resulting in CsgA–CsgB heteropolymers, while surface-anchored CsgB in MBP–CsgBII triggers morphologically distinct, CsgB-free/CsgA Bfco organelles. In the absence of CsgA, CsgB can self-assemble into polymers.

**Keywords**: assembly/curli organelle/immunoelectron microscopy/MBP–CsgB fusions/nucleator

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**Introduction**

Bacterial fimbriae are filamentous appendages, ranging from 2 to 8 nm in diameter on the surface of bacteria, that mediate bacteria–bacteria and bacteria–host cell interactions through their ability to bind specific structures on the cell surfaces (Duguid et al., 1955; Hultgren et al., 1991). In addition to classical fimbriae, many clinical *Salmonella* and *Escherichia coli* isolates as well as certain strains of *E. coli* K12, such as MC4100, express masses of thin, irregular and highly aggregated surface fibres denoted curli, when grown below 37°C, at low osmolarity during stationary phase (Olsen et al., 1989; Collinson et al., 1991; Sukupolvi et al., 1997). These curli fibres are morphologically distinct from the rigid Type I pili also formed by *E. coli* K12 (Brinton, 1965). Curliated bacteria bind a series of host proteins such as fibronectin, laminin, plasminogen and human contact phase proteins (Olsen et al., 1989; Arnqvist et al., 1992; Sjöbring et al., 1994; Ben Nasr et al., 1996). In addition, bacterial colonies of a curli-producing strain stain with the dye Congo red. So far, six csg genes located in a gene cluster at 23.8 minutes have been shown to be involved in the regulation and biogenesis of curli, and the functions of some gene products are now becoming clear. The major subunit protein CsgA is a 15.3 kDa protein encoded by csgA (Olsen et al., 1993). The co-transcribed csgB gene product shows high sequence homology to CsgA, with 49% similarity and 30% identity, and was therefore speculated to constitute a minor component of curli (Arnqvist et al., 1994), even though it was possible to identify only CsgA in a purified curli preparation (Collinson et al., 1991; Olsen et al., 1993). The genes csgD, csgE, csgF and csgG are all transcribed in the opposite orientation to the csgBA operon, and are involved in curli formation, since polar mutations in each of them abolish the expression of curli (Hammar et al., 1995). CsgD is a transcriptional activator required for the expression of csgA and other csg genes, while CsgE, CsgF and CsgG act at different levels in the subunit secretion process (our unpublished data).

The bacterial strategy to assemble fimbriae such as P and Type 1 fimbriae has been carefully studied (Hultgren et al., 1991). This pathway involves sec-dependent transport across the cytoplasmic membrane of pilus subunit proteins. To prevent premature interactions, subunit proteins are complexed to periplasmic chaperones. Subunit–chaperone complexes thereafter interact with an outer membrane usher, resulting in a release of the chaperone and subsequent polymerization by addition of subunits to the base of the growing fibres (Hultgren et al., 1991; Jones et al., 1993). Curli are assembled on the bacterial cell surface following a different pathway that involves a precipitation reaction of secreted soluble CsgA (Hammar et al., 1996). CsgB was found to be required for this process and was therefore thought to be located on the bacterial surface, acting as a nucleator for CsgA monomer, leading to the polymerization of curli fibres.

In this study, we identify the location and function of the CsgB protein for the assembly of wild-type curli. We show that the 110 C-terminal amino acids of CsgB fused to the C-terminus of maltose-binding protein (MBP) can substitute for the 131 amino acid native CsgB in the polymerization of soluble CsgA into surface organelles that are, however, morphologically different from wild-type curli. These so-called ‘Bfco’ organelles (CsgB-Free Curli-related Organelles) appeared thicker and more regular structures under the electron microscope as compared with wild-type curli. To explain the nature of this change in organelle morphology, immunoelectron microscopy was performed on wild-type and mutants overexpressing CsgB in the presence or absence of CsgA. The data suggest that...
in wild-type, CsgB, like CsgA, is secreted across the outer membrane. However, CsgB, unlike CsgA, is prone to be anchored at the cell surface after translocation. When overexpressed in the absence of CsgA, CsgB was able to self-assemble into short polymers on the bacterial surface. When both native proteins are expressed, CsgB is believed to impose a conformational alteration on CsgA by protein–protein interactions, leading to the CsgA-CsgB heteropolymeric wild-type curli fibres. When CsgB is anchored in the outer membrane as a fusion with the periplasmic MBP protein, only CsgA polymerization is triggered, leading to the formation of highly regular structures, CsgB-free/CsgA Bfco organelles, at one of the bacterial poles.

**Results**

**Surface-located CsgB is a minor component of wild-type curli and self-assembles into short polymers**

Previous studies showed that CsgA was the only detectable protein in a purified preparation of curli in *Salmonella* and *E. coli* (Collinson et al., 1991; Olsen et al., 1993). The deduced amino acid sequence of CsgB, however, shares high homology with CsgA (Arnvist et al., 1994). We therefore asked whether wild-type curli would not be only composed of CsgA but also CsgB. The *E. coli* strains MC4100 (*csgA*<sup>+</sup>, *csgB*<sup>+</sup>), MHR263 (MC4100 *recA, csgB*), MHR204 (MC4100 *csgA*) and MHR222 (MC4100 *csgA, csgB*) were therefore grown on YESCA plates (Hammar et al., 1995) under curli-proficient conditions for >48 h before performing immunoelectron microscopy and immunoblotting with the affinity-purified anti-CsgB peptide antibody ZB-αB.

As shown in Figure 1, curli expressed by MC4100 were decorated with gold particles when anti-CsgB antibody was probed (Figure 1A), suggesting that CsgB indeed is present in wild-type curli fibres. We also overexpressed CsgB by subcloning a full-length version of *csgB*, including the signal peptide, into the expression vector pKK223-3, resulting in pZB-KB1 (Table I), where *csgB* was expressed under the isopropyl-β-d-thiogalactopyranoside (IPTG)-inducible tac promoter. pZB-KB1 was next transformed into the *csgB*-deficient strain MHR263. Overexpression of CsgB in MHR263/pZB-KB1 resulted in an abundance of curli-like fibres on the cell surface. These fibres were labelled extensively by the gold particles when probed with anti-CsgB antibody (Figure 1B), indicating that more CsgB proteins are present in these fibres than in wild-type curli. Interestingly, gold particles frequently were located at sites where curli fibres appeared to branch (Figure 1B2–B4).

As shown in Figure 2, CsgB protein was detected by immunoblot in wild-type strain MC4100 (Figure 2, lanes 1– and 2+), but not in the negative control MHR263 (Figure 2, lane 7–). Curli fibres are highly insoluble in SDS (Collinson et al., 1991; Olsen et al., 1993), resulting in the presence of high oligomeric forms in SDS–PAGE stacking gels without prior treatment with 90% formic acid (Hammar et al., 1996). In MC4100, an extra signal was detected by anti-CsgB antibody at the top of the stacking gel, indicating that part of CsgB was present in a polymer which, like CsgA in curli fibres, is resistant to SDS treatment during the electrophoresis (Figure 2, lane 1–). For MHR263/pZB-KB1, more SDS-insoluble material appeared at the top of the stacking gel (Figure 2, lane 3–) analysed by anti-CsgB antibody compared with that of wild-type strain MC4100 (Figure 2, lane 1–). We also treated the cells of wild-type curli-expressing strain MC4100 and CsgB-overexpressing strain MHR263/pZB-KB1 with 90% formic acid prior to SDS–PAGE. Large amounts of monomeric CsgB were found in the separating gel that reacted with ZB-αB (Figure 2, lanes 2+ and 4+) compared with the same amount of cell lysis sample without prior formic acid treatment (Figure 2, lanes 1– and 3–), indicating that CsgB subunits are incorporated into wild-type curli fibres and even more so when being overexpressed. Like the major subunit protein CsgA, the minor subunit protein CsgB requires formic acid treatment for depolymerization, arguing that CsgB is integrated into the curli fibres and not merely associated with their exterior surface.

In MHR204 lacking the major subunit CsgA and expressing wild-type levels of CsgB, the bacterial surface was stained with more gold particles per area than the surroundings (Figure 3A) when CsgB-specific antibody was used for immunolabelling, suggesting that CsgB is exposed on the bacterial cell; however no surface structures
were seen. When pZB-KB1 was introduced into MHR222, a strain unable to express CsgA as well as CsgB due to a polar insertion in csgB, colonies of MHR222/pZB-KB1 were stained with Congo red on a YESCA agar plate under curli-proficient condition (data not shown). Large quantities of cottony growth on the surface of the bacterial cell were seen under the electron microscope. No distinct structure was detected at lower magnification (Figure 3B). However, at high magnification, short polymers were clearly seen (Figure 3C) that reacted with the anti-CsgB antibody and therefore were decorated with gold particles. Nevertheless, short polymers of CsgBII where the CsgB portion lacks the 20 amino-terminal amino acids of the mature protein. The E.coli strain MHR263, lacking expression of native CsgB, was transformed with pZB-MB2 and pMAL-p2 respectively, generated a 339 bp PCR fragment corresponding to codons 21–131 of the csgB gene of MC4100, and fused it to the malE gene of pMAL-p2, resulting in pZB-MB2 (Table II, Figure 2). This plasmid codes for a fusion protein MBP–CsgB fusion protein triggers in vivo assembly of Bfco organelles that are morphologically different from wild-type curli

An MBP–CsgB fusion protein triggers in vivo assembly of Bfco organelles that are morphologically different from wild-type curli

In order to prevent CsgB secretion and assembly into curli, we attempted to anchor CsgB in the outer membrane by fusing it to the periplasmic MBP. To this end, we generated a 339 bp PCR fragment corresponding to codons 21–131 of the csgB gene of MC4100, and fused it to the malE gene of pMAL-p2, resulting in pZB-MB2 (Table I, Figure 2). This plasmid codes for a fusion protein MBP–CsgBII which remains in the slot reacting with the anti-CsgB antibody and therefore was decorated with gold particles. Membrane and analysed with CsgB-specific antibody ZB–αCsgA polyclonal Ab Hammar et al. (1996)

Western immunoblots of native CsgB and overexpressed CsgB proteins were separated by 15% SDS–PAGE, transferred onto blotting membrane and analysed with CsgB-specific antibody ZB–αCsgB peptide Ab this work

CsgB nucleator function in curli assembly

Fig. 2. Western immunoblots of native CsgB and overexpressed CsgB in E.coli. MC4100, MHR263/pZB-KB1, MHR222/pZB-KB1 and MHR263 were grown on YESCA plates for >48 h at 28°C. Cells were loaded with (+) or without (–) prior formic acid treatment, and proteins were separated by 15% SDS–PAGE, transferred onto blotting membrane and analysed with CsgB-specific antibody ZB–αCsgB peptide Ab this work

CsgB nucleator function in curli assembly

Table 1. Escherichia coli strains, plasmids and antibodies used in this study

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Fig. 3. Electron micrographs of cell surface-exposed CsgB in the csgA mutant strain MHR204 (A) and short CsgB polymers in MHR222/pZB-KB1 (B, C and D). Bacteria were grown on YESCA plates with or without carbenicillin under curli-proficient conditions for 48 h. Cells were either only negatively stained with 0.2% uranyl acetate (B and C) or combined with immunolabelling (A and D). Cells of MC4100 and MHR222/pZB-KB1 (A and D) were incubated with CsgB-specific antibody ZB-αB. Bacterial surface structures were labelled with protein A–gold particles (10 nm in diameter). Magnification: (A) ×100 000; (B) ×30 000; (C) ×200 000; (D) ×60 000.

Fig. 4. The csg gene cluster and derived CsgB protein derivatives. Arrows indicate the position and direction of the two csg promoters. Genes encoding proteins assumed to be exported are marked by boxes with closed promoter-proximal ends. The leftmost column shows the CsgB derivatives. The middle column shows their relative sizes; the repeated stars above the full-length CsgB show the positions of the 3×22 and 1×19 amino acid repeat region; the repeated short vertical bars above the MBP-CsgBIX indicate the location of the peptides, CsgB-PEP2 and CsgB-PEP1, against which the anti-CsgB peptide antiserum was raised. SP indicates the signal peptide. The rightmost column indicates the results of the complementation experiment in the csgB mutant MHR263.

Conditions without antibiotic and inducer. After 36 h of incubation, colonies of MC4100 and MHR263/pZB-MB2 turned red due to binding of the dye Congo red present in the YESCA plates. However, MHR263/pZB-MB2 colonies were stained less red than those of MC4100 (data not shown). Since curli expression is associated with a Congo red-binding phenotype, we speculated that strain MHR263/pZB-MB2 might be able to produce curli-related surface structures. To test this, bacteria harvested after 48 h were examined by electron microscopy (Figure 5). Organelles were present in MHR263/pZB-MB2 (Figure 5A) but not in MHR263/pMAL-p2 (Figure 5D). Strikingly, these organelles were morphologically very different from the curli fibres expressed by wild-type strain MC4100 (Figure 1). At high magnification, the organelles on MHR263/pZB-MB2 appeared as distinctly curved and loosely aggregated, 10–15 nm thick (the gold particles provided a standard of 10 nm in diameter) highly ordered fibres (Figure 5B, C and E), in contrast to the irregular branching masses of much less distinct, and much thinner (2 nm) wild-type curli fibres (Figure 1). The morphologic-
ally novel surface organelles on MHR263/pZB-MB2 were decorated symmetrically with gold particles along their length (Figure 5B) when the intact cells were incubated with the affinity-purified CsgA-specific antibody ZB-αA (Hammar et al., 1996), but not with CsgB-specific antibody ZB-αB (Figure 5C) or an anti-MBP monoclonal antibody (Figure 5E).

In a Western blot, in vivo expressed MBP–CsgBII interacted with anti-MBP serum (Figure 6). When expressed in the csgA, csgB mutant strain MHR222, MBP–CsgBII was totally soluble in SDS (Figure 6, lane 1). However, when the same fusion protein was expressed in the CsgA-expressing strain, MHR263, under the same conditions, some MBP–CsgBII remained in the slot (Figure 6, lane 2). MHR263/pMAL-p2 was analysed as a control shown in Figure 6, lane 3. These data suggest that MBP–CsgBII by itself is unable to form SDS-resistant polymers but can stably interact with polymerized CsgA. Also, the fusion protein expressed in the absence of CsgA appeared to be more susceptible to in vivo proteolytic degradation.

Since the organelles observed were produced by a strain lacking a functional csgB gene, and they were labelled with anti-CsgA but not anti-CsgB antibodies, we interpret these novel organelles as being built up of CsgB subunits, free from CsgB protein, namely Bfco organelles.

**MBP–CsgBII localizes to one of the two bacterial poles as do Bfco organelles**

Unlike wild-type curli fibres, Bfco organelles resulting from the presence of CsgB fused to MBP appeared at only one of the two bacterial poles (Figure 7). Since it has been demonstrated that MBP is located predominantly in the periplasmic space at the bacterial pole (Dietzel et al., 1978; Boos and Staehelin, 1981; Maddock and Shapiro, 1993), we wondered if polar assembly of Bfco organelles was due to polar location of MBP–CsgBII. As shown in Figure 5C, the poles containing Bfco organelles were also specifically surface labelled with gold particles when the ZB-αB antibody was probed. In contrast, no surface labelling was seen when the anti-MBP monoclonal antibody was used (Figure 5E), suggesting that the CsgB portion in the fusion is exposed on the cell surface, whereas its fusion partner MBP is not. Since MBP is a known periplasmic protein, it may be that this portion of the fusion protein is retained in the periplasm because of its bulky form. Thus, MBP–CsgBII goes to one of the two bacterial poles where the CsgB portion of the fusion protein can traverse the outer membrane and act as a membrane-bound nucleator for CsgA polymerization into Bfco fibres.

**Fibronectin-binding properties of fibres containing CsgA, CsgB or both proteins**

Curliated *E.coli* bind fibronectin while *E.coli* knockout mutants in either csgA or csgB do not. Depolymerized curli subunit proteins also bind fibronectin in a filter binding assay (Olsen et al., 1993). Since CsgB is predicted to have a molecular weight similar to CsgA, and in view of the present finding that CsgB is a constituent of wild-type curli, it is possible that CsgB alone or in addition to CsgA contributes to fibronectin binding. A non-radiolabel assay of fibronectin binding to *Staphylococcus aureus* has been shown to yield a satisfactory signal-to-background ratio, which was comparable with a radiolabel assay (Flock et al., 1990). In this study, we used the same method with some modifications, described in Materials and methods. As shown in Figure 8, *E.coli* MHR263/pZB-KB1 producing curli with a higher content of CsgB bound fibro-
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Fig. 8. Binding of *E. coli* to plastic coated with fibronectin. Bacterial cells were grown on YESCA plates under curli-proficient conditions for >48 h. Microtitre wells were coated with 100 μl of bovine fibronectin at concentrations ranging from 0.08 to 10 μg/ml. Two hundred μl of 4×10^8 cells/ml bacteria (OD 600 0.94–0.95) were added. Adherent bacteria were determined with a microplate reader (A_{405}). Each value is the mean and standard error of one (MHR204, MHR263/pMAL-p2) or two (MC4100, MHR263/pZB-KB1, MHR222/pZB-KB1, MHR263/pZB-MB2) independent triplicate assays. (A) The relative adherence of bacteria from each strain binding to fibronectin at different concentrations. (B) Two hundred μl of 4×10^8 cells/ml of bacteria from each strain binding to fibronectin at the concentration of 5 μg/ml.

Fig. 9. SDS–PAGE chromatography of affinity-purified MBP and MBP-CsgB fusion proteins expressed in *E. coli* XL1-Blue. Bacterial cells harbouring pMAL-p2, pZB-MB2, pZB-MB3, pZB-MB4, pZB-MB8 and pZB-MB9 were grown in LB medium with 50 μg/mg carbenicillin. The fusion proteins were induced with 0.3 mM IPTG and affinity-purified as described in Materials and methods. Proteins were subjected to 15% SDS–PAGE and stained with Coomassie brilliant blue. MBP (lane 1) is the standard with a mol. wt of 42 kDa.

The C-terminal 110 amino acid pseudo repeat region is essential for triggering polymerization of CsgA in vivo

To localize further the region of CsgB capable of triggering the polymerization of CsgA, various portions of csgB were amplified and cloned into pMAL-p2, yielding a series of malE::csgB fusion derivatives (Table I, Figure 4). MBP and the fusion proteins (MBP–BII, MBP–BIII, MBP–BIV, MBP–BVIII and MBP–BIX) were expressed in the host *E. coli* strain XL1-Blue after induction with 0.3 mM IPTG. Thereafter, the respective fusion proteins were affinity purified on an amylose resin column. The purified products of these malE::csgB fusion clones were run on a 15% SDS–polyacrylamide gel as shown in Figure 9. MBP has an Mₘ of 42 kDa (Figure 9, lane 1), whereas the individual fusion proteins were all larger, but protein degradation occurred for some of them, resulting in multiple protein bands. The malE::csgB fusion plasmids were transformed into the csgB mutant strain MHR263, and the resulting transformants incubated on YESCA plates with carbenicillin at 28°C for >48 h. Except for MHR263/pZB-MB2, no other strains gave rise to Congo red-binding colonies and no surface structures were visible under the electron microscope (data not shown). Western immunoblotting assays verified that the fusions reacted with both anti-MBP monoclonal antibody and anti-CsgB antiserum (Figure 10). However, protein degradation occurred in all cases. Assuming that the largest immuno-reactive protein band for MBP–CsgBIII (Figure 10, lane 2) corresponds to the full-length nucleator active version of the fusion, the largest bands for MBP–CsgBIII and MBP–CsgBVIII (Figure 10, lanes 3 and 5) run with a molecular weight corresponding to the complete fusion proteins. Low amounts of the apparent full-length protein were also observed for in vivo expressed MBP–CsgBIV.
and MBP–CsgBVIII (Figure 10, lanes 4 and 6). Therefore, all the fusion proteins were expressed in MHR263. Since MBP–CsgBVIII lacks 23 N-terminal CsgB residues relative to MBP–CsgBII, and MBP–CsgBIII lacks 43 C-terminal CsgB residues, we conclude that most of the 110 CsgB residues in MBP–CsgBII are needed for triggering the polymerization of CsgA. This region of CsgB in MBP–CsgBII contains the complete set of pseudo repeats also seen in CsgA (Hammar et al., 1996).

**Discussion**

We recently have demonstrated that the assembly of curli fibres in *E. coli* occurs outside the microbe through precipitation of secreted soluble CsgA subunit protein into thin aggregated fibres (Hammar et al., 1996). This reaction requires the CsgB protein that shows extensive sequence homology to CsgA. It was suggested that CsgB acts as a nucleator for the polymerization of soluble CsgA monomer, since these fibres were formed intercellularly on cells expressing only CsgB, provided such cells were grown in close proximity to cells lacking CsgB, and therefore secreting soluble CsgA. To explain this inter-bacterial fibre formation, we speculated that CsgB might be surface exposed on the bacteria, acting as a nucleator or an anchor.

In this study, by using immunoelectron microscopy and immunoblotting assays with the affinity-purified specific anti-CsgB and anti-CsgA antibodies, ZB-αB and ZB-αA, we demonstrate the capacity of CsgB to act as a nucleator, triggering the polymerization of CsgA monomers, forming wild-type curli fibres and Bfco organelles. We show that CsgB expressed at wild-type levels in the absence of CsgA is located on the bacterial cell surface and, when expressed together with CsgA, it also appears along the length of the curli fibres. When CsgB was overexpressed, more CsgB molecules were present in the curli-like fibres without changing the morphology compared with wild-type curli. Interestingly, in the absence of CsgA, over-expression of CsgB resulted in the formation of SDS-insoluble short CsgB polymers on the bacterial cell surface.

We suggest that, at wild-type levels, CsgB is translocated across the outer membrane, after which it can either bind to components on the exterior surface of the outer membrane or be engaged in polymerization with CsgA. We assume that the translocation of CsgB and CsgA across the outer membrane requires a specific protein translocator, the nature of which is unknown. It is possible that the concomitant translocation of CsgB and CsgA through the same translocator may allow CsgB-mediated nucleation during the secretion process, allowing CsgB to be pushed out from the cell surface by polymerized CsgA. CsgB in the polymerized fibre was often found at sites where curli fibres appeared to branch. Such branching would be possible to explain if CsgB contains multiple interaction sites with CsgA or if oligomeric forms of CsgB allow for multinucleation. Hence, when CsgB proteins are present in wild-type curli, the free site of an individual CsgB molecule or CsgB oligomer might act as a new nucleation site, triggering the polymerization of soluble CsgA, resulting in the formation of highly branched fibres.

Nucleation events may also occur with surface-bound CsgB for bacterial anchorage of the curli fibres.

When CsgB is fused to MBP, we believe that the bulky MBP portion of the fusion protein cannot pass through the outer membrane translocator, leading to a transmembrane protein where only the CsgB portion is surface exposed. Such a surface-anchored nucleator can only trigger polymerization of CsgA molecules secreted through separate translocators, only allowing the growth of CsgA fibres from the distal end without the possibility of branching.

The presence of MBP in MBP–CsgBII not only results in the formation of highly ordered Bfco organelles lacking incorporated CsgB, but also affects the position of fibres on the bacterial cell. Thus, the Bfco fibres only appeared at one of the two bacterial poles. We believe that this polar location is due to a polar secretion of MBP–CsgBII, as has been shown for MBP alone (Dietzel et al., 1978; Boos and Staehelin, 1981; Maddock and Shapiro, 1993). Asymmetric distribution of a number of bacterial proteins has been described in recent years (Kocks et al., 1993; Shapiro and Losick, 1997). This asymmetry does not seem to depend on random insertion followed by non-random protein translocation, but rather on an asymmetric targeting of proteins within the cell. In the present study, we argue that MBP but not CsgB is targeted for polar secretion across the cytoplasmic membrane, causing the positional difference between Bfco organelles and wild-type curli fibres.

We speculate that the binding of MBP–CsgBII to CsgA induces CsgA to undergo a structural transition to a conformation that mimics that of CsgB. The conformationally altered CsgA is now able to bind the next soluble CsgA monomer and induce a similar conformational change. A repetition of this process eventually leads to the formation of long insoluble CsgA-containing Bfco organelles. The above model bears similarity with the proposed process through which the scrapie protein PrPSc, by binding to PrPC, induces a conformational change of PrPC (Glenn et al., 1996; Huang et al., 1996).

Biological structures frequently contain assemblies or complexes of several protein subunits. The high sequence homology between CsgA and CsgB suggests that both proteins possess some similar structural and functional characteristics for the formation of curli fibres. The finding here that CsgB is another component of curli is contrary to the original observation that CsgA was the only subunit protein found in a purified curli preparation (Collinson et al., 1991; Olsen et al., 1993). It is possible that CsgB is lost during the harsh curli preparation. Alternatively, CsgB might normally be present in curli in such low amounts that its presence was not detected, and was simply hidden beneath the CsgA protein band after depolymerization in 90% formic acid. The present data clearly demonstrate that CsgB is integrated into curli as a minor component, and not merely associated with the exterior surface of the fibres. Minor proteins are known to be present also in various fimbriae. In P fimbriae, these minor proteins are located at the tip of the organelles, fulfilling functions as adhesins, adaptors or possibly initiators for polymerization (Lindberg et al., 1987; Kuehn et al., 1992; Jacob-Dubuisson et al., 1993). In Type 1 fimbriae, the
FimH adhesin was found both at the tip and along the pilus fibres (Ponniah et al., 1991; Jones et al., 1995).

Even though CsgA and CsgB show 30% identity and 49% similarity, and are built up of similar repeat motifs, they clearly behave quite differently. In the absence of CsgB, CsgA is secreted into the medium (Hammar et al., 1996) and, even when it is overexpressed, no autoaggregates can be observed on the bacterial surface or in the culture medium (data not shown). CsgB is also translocated through the outer membrane, but is not secreted into the medium in the absence of CsgA, even when overexpressed (data not shown). It is possible that in the presence of CsgA, CsgB is secreted, but incorporated immediately into fibres due to its nucleating activity. Immunoelectron microscopy shown here reveals that CsgB is surface exposed in the absence of CsgA. This also has been verified recently by Loferer et al., 1997, where CsgB was found to be completely sensitive to proteinase K treatment of intact cells. Overexpression of CsgB in the absence of CsgA resulted in masses of short polymers on the bacterial surface. The polymers were SDS resistant and therefore reacting with CsgB-specific antibody at the top of the stacking gel. In contrast, CsgA expressed in the absence of CsgB remained completely SDS soluble. Thus, CsgB and CsgA are both translocated. However, CsgB produced in the absence of CsgA is prone to self-assemble, leading to a precipitation on the bacterial surface, whereas CsgA depends on CsgB for precipitation into curli fibres. It is intriguing but not all surprising that CsgB can act both as a nucleator protein for CsgA and by its itself polymerize into polymers. Recently, γ-tubulin was demonstrated to be both a nucleator for microtubule formation and to self-assemble into novel tubular structures in mammalian cells (Shu and Joshi, 1995). γ-tubulin is a new member of the tubulin gene family with up to 35% sequence identity to the classical α- and β-tubulins (Joshi et al., 1992).

If CsgB acts as a true nucleator, it must be able to interact with CsgA. The sequence homology between CsgB and CsgA makes it likely that CsgB physically interacts with CsgA. Our evidence for such a direct interaction is as follows: (i) when CsgB is produced by itself at wild-type levels it is surface located, but when expressed together with CsgA in wild-type bacteria it is present in curli fibres, suggesting a CsgB–CsgA interaction; (ii) the MBP–CsgBII fusion protein was stabilized when CsgA was produced, and appeared in an SDS-resistant complex at the top of the gel only in the presence of CsgA; (iii) the polar expression of the nucleator CsgB as part of the MBP–CsgBII fusion results in polar assembly of CsgA-containing Bfco organelles.

The MBP–CsgBII fusion contains the 110 C-terminal residues of CsgB. The 20 N-terminal residues of mature CsgB are therefore not required for the nucleation function. These 110 amino acids contain the entire repeat region of the native CsgB. Since fusion protein MBP–CsgBVIII, lacking only an additional 23 amino acids from the N-terminal region including the first repeat, is totally inactive as a nucleator, we suggest that a complete repeat region is necessary for nucleator function. Since CsgA contains a very similar repeat region, it is reasonable to hypothesize that an interaction between the repeat regions of CsgB and CsgA induces a conformational change in the latter protein leading to the formation of surface organelles.

Hydrophobic interactions are regarded as being important in a variety of microbial adhesion phenomena in both the environment and the host (Rosenberg and Doyle, 1990). Congo red binding has been used as an indication of surface hydrophobicity for certain pathogens (Surgalla and Beesley, 1969). In some strains of E.coli, cell surface hydrophobicity has been related to the presence of fimbriae (Ljungh and Wadstrom, 1982; Wadstrom et al., 1986). Congo red binding has been shown to be tightly associated with the expression of curli fibres in E.coli (Hammar et al., 1995). We have shown here that the structural properties of both CsgA-containing Bfco organelles and the short CsgB polymers are associated independently with a Congo red-binding phenotype, indicating that surface structures resulting from CsgA, free from CsgB, and CsgB, free from CsgA, are able to bind this dye. We do not know whether other csg gene products besides CsgA and CsgB may contribute to the Congo red-binding phenotype.

Fibronectin binding is one of the unique features of curli-expressing E.coli and Salmonella (Olsen et al., 1989; Collinson et al., 1991). Besides fibronectin, curli-expressing bacteria bind a series of host proteins such as laminin, plasminogen and human contact phase proteins (Arnqvist et al., 1992; Sjöbring et al., 1994; Ben Nasr et al., 1996). The present data clearly demonstrate that binding of curli-expressing E.coli to fibronectin is influenced by the presence of CsgB protein in the fibres, because overexpression of CsgB in the presence of CsgA results in a higher level of fibronectin binding as compared with bacteria expressing wild-type curli. Cells expressing Bfco organelles or short CsgB polymers also bind fibronectin, but at a much lower level, suggesting that bacterial surface structures made up of either CsgA or CsgB were able to bind fibronectin. However, since cells expressing wild-type curli show a much higher fibronectin-binding capacity than cells expressing Bfco organelles, this indicates that CsgB, as an integral part of curli, contributes to fibronectin binding. Previous studies have shown that depolymerized curli monomers can bind fibronectin (Olsen et al., 1993). Since CsgA, prior to this study, was thought to be the sole component of curli, it was suggested that binding was to monomeric CsgA. As the CsgB protein has the same molecular weight as CsgA, it is a minor component of wild-type curli and can be depolymerized into monomers by formic acid treatment, fibronectin binding might be to monomeric CsgB.

To understand fully how CsgB mediates its nucleation function on CsgA and how this protein contributes to the adhesive property of curli, in vitro assembly from purified components is needed. However, before such studies can be initiated, it is necessary to characterize other csg genes involved in the formation of curli and see if their gene products may affect either CsgA or CsgB post-translationally.

Materials and methods

Experimental materials

Restriction enzymes and DNA-modifying enzymes were used as recommended by the manufacturer (Boeringher Mannheim, New England...
Table II. Oligonucleotides used as primers in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Position in csg gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNII-73</td>
<td>5'-GGCGGAGAAAGCTTCTTATT-3'</td>
<td>5349</td>
</tr>
<tr>
<td>BCI-74</td>
<td>5'-TGCATAAGCTAAGCTTGGTG-3'</td>
<td>3688</td>
</tr>
<tr>
<td>BI-401</td>
<td>5'-GCTATGATCCAGGCAGCTCG-3'</td>
<td>3558</td>
</tr>
<tr>
<td>BCI-402</td>
<td>5'-TGCAAAGGCCTACTGTCGCT-3'</td>
<td>3495</td>
</tr>
<tr>
<td>BNIVIII-4601</td>
<td>5'-GGCGGAGAAGCCTCAAACTTGGG-3'</td>
<td>3424</td>
</tr>
<tr>
<td>BNIX-4602</td>
<td>5'-ATGCAAGATTCTCCGAAAGTG-3'</td>
<td>3538</td>
</tr>
<tr>
<td>BNVIII-402</td>
<td>5'-CGGAATTCAGGGGTCGACACAT-3'</td>
<td>3218</td>
</tr>
<tr>
<td>Malе*</td>
<td>5'-GGTCGTCAGCTGATGAGACC-3'</td>
<td>tac-trp-605</td>
</tr>
<tr>
<td></td>
<td>5'-TGAGCTGTGGACATAAATCTACG-3'</td>
<td></td>
</tr>
</tbody>
</table>

*aThe csg sequence is available from DDBJ/EMBL/GenBank under the accession No. x09754.

Bacterial strains, plasmids and antibodies

The E. coli strains, plasmids and antibodies used in this study are described in Table I.

Construction of plasmids

Oligonucleotide primers used to amplify different portions of the csgB gene are listed in Table II. PCR was carried out in a 100 μl reaction containing 50 pmol of each primer and 250 μM of each dNTP in Taq reaction buffer [10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl2, 50 mM KCl]. Crude chromosomal DNA isolated from wild-type strain MC4100 was used as template. Amplification was performed on an automatic thermocycler (Perkin Elmer Cetus). Each reaction was electrophoresed through a 1.5% agarose gel to verify production of the appropriate fragments. The PCR products were excised and purified using GeneClean (BioRad). PCR products used for the constructs (Table I) were digested sequentially with restriction enzymes. Following heat inactivation and phenol extraction, the digested PCR products were purified with GeneClean again. Plasmids pMAL-p2 and pKK223-3 were digested sequentially and purified. Ligation reactions were carried out using standard protocols (Miller, 1972). Transformants were screened by plating duplicates on LB agar with 100 μg/ml carbenicillin, 25 μg/ml X-gal and 0.25 mM IPTG, when working with pMAL-p2. Candidate colonies were checked by PCR with the appropriate primers. Plasmid DNA was isolated from the positive clones and determined by densitometry of ethidium bromide staining in agarose gel (Sequenase). Oligonucleotides used for sequencing were primers of Malе* for malе fusion constructs (pZB-MB2, pZB-MB3, pZB-MB4, pZB-MB5 and pZB-MB9) and tac-trp-605 for pZB-KB1.

Purification of MBP and MBP–CsgB fusion proteins

MBP and fusion proteins were purified by affinity chromatography. An overnight broth culture of E. coli strain XL1-Blue, harbouring pMAL-p2, pZB-MB2, pZB-MB3, pZB-MB4, pZB-MB5 and pZB-MB9 respectively, was inoculated into 100 volumes of LB broth plus 50 μg/ml carbenicillin, and incubated at 37°C until the OD600 reached 0.6. The expression of proteins was induced by 0.3 mM IPTG, and bacterial cells were incubated for an additional 2 h. Cells were pelleted by centrifugation and the periplasmic contents containing the fusion protein were released by osmotic shock treatment (Neu and Heppel, 1965). One volume of 5% column buffer [100 mM Tris–HCl (pH 7.4), 1 M NaCl, 5 mM EDTA, 50 mM 2-mercaptoethanol, 5 mM Na2SO4] was added to four volumes of the supernatant (osmotic shock fluid). The buffered osmotic shock fluid was applied to a column ( Econo-Column, 1.5 x 5 cm, Bio-Rad) packed with amylose resin at 0.5 ml/min. After intensive washing with column buffer, MBP and MBP–CsgB fusion proteins were eluted with 10 mM maltose and collected into fractions. An aliquot from each fraction was examined by electrophoresis through a 15% SDS–polyacrylamide gel. Proteins were visualized by Coomassie blue staining.

Purification of polyclonal antibodies

ZB-tα. About 10 ml of CNBr-activated Sepharose 4B gel were prepared according to the supplier (Pharmacia Biotech, Sweden). Twenty mg of MBP were coupled onto the beads by incubating overnight at 4°C in coupling buffer [0.1 M NaHCO3 (pH 8.3), 0.5 M NaCl]. The adsorbent was packed into a low-pressure column (C-column, Pharmacia Biotech). Ten ml of rabbit antiserum raised against MBP–CsgAIII (Hammar, 1996) was passed down the column at 6 ml/h. All the sera which could not be adsorbed were collected into fractions. Antiseras in the fractions were again adsorbed extensively against the lysates from E. coli strain MC4100 grown at 37°C, and therefore not expressing csg genes products, resulting in the specific anti-CsgB polyclonal antibodies. ZB-βα (ZB-AII, Hammar et al., 1996). ZB-αα was used for immunoelectron microscopy and immunoblotting at a dilution of 1:200–1:2000.

ZB-αβ. Two peptides were designed according to the deduced amino acid sequence of the csgB gene (CsgB-PEP-PA, aa 54–69 and CsgB-PEP-PA, aa 111–131). The peptides were synthesized commercially and used to immunize rabbits (TANA Lab, Texas). Ten ml of CNBr-activated Sepharose 4B gel were prepared according to the supplier (Pharmacia Biotech, Sweden). Two ml of the mixture of the two peptides were coupled to the beads by incubation overnight at 4°C in coupling buffer [0.1 M NaHCO3 (pH 8.3), 0.5 M NaCl]. The adsorbent was packed into a low-pressure column (C-column, Pharmacia Biotech). Five ml of rabbit antiserum were passed down the column at 0.25 ml/min. After intensive washing with phosphate-buffered saline (PBS), the specific anti-CsgB immunoglobulins, ZB-βα, which bound to the beads were eluted with glycin, HCl, NaCl buffer, and collected into fractions which were neutralized with Tris base buffer. ZB-αβ was used for immunoelectron microscopy and immunoblotting at a dilution of 1:50–1:500.

Immunoblot

Bacterial cells were incubated on YESCA plates (Hammar et al., 1995) for >48 h at 28°C. A suspension of bacterial cells in protein sample buffer was boiled for 10 min, and the constituent proteins were separated on a 15% SDS–polyacrylamide slab gel (Swank and Munkres, 1971). Immunoblot experiments were performed essentially as described earlier (Towbin et al., 1979). The proteins were then transferred onto a nitrocellulose membrane (0.45 μm pore size in roller form, MSH, USA) at 100 V for 60 min. The electrophoretic blot was soaked in 5% non-fat milk in TBS [10 mM Tris–HCl (pH 7.5), 150 mM NaCl] for at least 30 min. The membrane was incubated with purified ZB-αα at 1:1000–1:2000 dilution and anti-MBP monoclonal antibody at 1:10 000 dilution in TBS containing 0.5% bovine serum albumin (BSA) for 60 min at 37°C. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Dakopatts AB) at 1:4000 dilution was used as the second antibody. Coloured alkaline phosphatase products on the nitrocellulose sheet were developed with NBT/BCIP in alkaline phosphatase buffer [0.1 M Tris–HCl (pH 9.5), 0.1 M NaCl, 5 mM MgCl2] at room temperature.

Electron microscopy

Electron microscopy was performed using Philips CM200, JOEL-1200 and Zeiss EM109 electron microscopes at 60–80 kV with copper or nickel grids (200 and 300 mesh) coated with thin films of 2% Formvar. Bacterial colonies after 48 h incubation were overlaid with PBS (pH 7.4), and the cell suspension was allowed to sediment for 2 min on a grid. After washing with PBS followed by distilled water, the specimen was negatively stained with 0.2% uranyl acetate and air dried before performing the electron-microscopic study. For immunoelectron microscopy, specimens were blocked with 1% BSA/PBS, and then incubated with ZB-αα (1:200 dilution in 1% BSA/PBS), ZB-βα (1:50 dilution) or anti-MBP antiserum (1:1000) for 60 min at 37°C. Following washing with PBS, the specimen was incubated with gold particle-labelled protein A (10 nm colloidal gold particles) for 30 min at 37°C, rinsed with PBS followed by distilled water and negatively stained.

Assay of fibronectin binding

Quantiﬁcation of non-radiolabelled ﬁbronectin binding to E.coli has been modiﬁed according to the method described earlier (Flock et al., 1996). Bacteria were grown on YESCA agar plates under curli-proficient conditions for 48 h. Marine agar plates (Difco, Bacto-Sea, Inc., Chantilly, VA) were coated with 100 μl of bovine ﬁbronectin (Sigma Chemical Co., St. Louis, MO), ranging from 0.08 to 10 μg/ml, at 4°C overnight and at room temperature for an additional 2 h. After blocking with 2% BSA in PBS (PBS with 0.05% Tween-20) for 2 h at room temperature, 200 μl of 4 x 10^5 cells/ml (OD600 = 0.94–0.95) in PBS

CsgB nucleator function in curli assembly
were constantly added to the wells and incubated at room temperature for 3 h. The wells were washed, and the amount of light absorbance of the adherent bacteria was determined with a microplate reader (A405).

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References


Surgalla,M.J. and Beesley,E.D. (1969) Congo red-agar plating medium in setting up the fibronectin-binding assay. Professor T.Hökkfelt (Karolinska Institute) is acknowledged for allowing use of their JOEL-1200 electron microscope for some of the EM work. Dr J.A.Ampofo (Institute of Aquatic Biology, Ghana) is acknowledged for reading the manuscript. Z. Huang is supported by a PhD scholarship from the Karolinska Institute. This work was supported by grants from the Swedish Medical Research Council (16x-10843), the Swedish Natural Science Research Council (3373-309), the Göran Gustafssons Foundation of Natural and Medical Science and an unrestricted grant from Bristol-Myers Squibbs to S.N.