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Nucleator-dependent intercellular assembly of adhesive curli organelles in *Escherichia coli*

(pilus/self-assembly/Congo red binding)

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ABSTRACT **Bacterial adhesion to other bacteria, to eukaryotic cells, and to extracellular matrix proteins is frequently mediated by cell surface-associated polymers (fimbriae) consisting of one or more subunit proteins. We have found that polymerization of curlin to fimbriae-like structures (curli) on the surface of *Escherichia coli* markedly differs from the prevailing model for fimbrial assembly in that it occurs extracellularly through a self-assembly process depending on a specific nucleator protein. The cell surface-bound nucleator primes the polymerization of curlin secreted by the nucleator-presenting cell or by adjacent cells. The addition of monomers to the growing filament seems to be driven by mass action and guided only by the diffusion gradient between the source of secreted monomer and the surface of monomer condensation.**

Many biological macromolecules are polymeric assemblies of a single kind of protein subunit. The polymerization is a self-assembly process of monomers forming tubular structures of helical or cylindrical configuration. Among the systems most studied are the tobacco mosaic virus, the bacterial flagellar filament, and the actin filament. These structures can be reconstituted *in vitro* from purified monomers (coat protein, flagellin, and G-actin, respectively) without the need of any external organizers, except for a nucleating agent, such as salts or "seeds," to initiate the polymerization (1–3). Structures such as these, which are self-assembled, of helical symmetry, and built of identical units connected by (almost) identical bonds, can be regarded as finite-sized surface crystals (4). The equilibrium feature of the transition from dispersed monomers to helical aggregates is an essential element in common with a crystallization or a condensation phenomenon and can be given a thermodynamic interpretation (5, 6). *Escherichia coli*, *Salmonella enteritidis*, and *Salmonella typhimurium* produce surface-bound, long, thin, flexible filaments called curli in *E. coli* (7) and thin, aggregative fimbriae or SEF 17 fimbriae in *Salmonella* (7) as a response to limiting nutrients. These organelles mediate plasminogen binding and activation (8), binding to fibronectin (9, 10), and bacterial autoaggregation (10). Curli may also bind the dye Congo red since the expression of curli is concomitant with the development of a dark red color of the colonies when they are grown on YESCA agar plates containing the Congo red (10, 11). When curli and SEF 17 fimbriae have been purified, only one protein has been recovered from each structure: curlin (CsgA), with a molecular mass of 15.3 kDa and encoded by the *csgA* gene (12), and the highly homologous AgfA subunit, with a molecular mass of 17 kDa and encoded by the *agfA* gene (7, 13), respectively. In *E. coli*, *csgA* is cotranscibed with *csgB*, encoding a polypeptide homologous to curlin. Another operon, *csgDEFG*, is transcribed in the opposite direction, upstream of the *csgBA* operon (←*GFED BA*→). All of these genes are necessary for production of curli, but their functions are unknown except for *csgD*, which is required for transcription of the *csgBA* operon (11). In this report, we demonstrate an intercellular phenotypic complementation of a *csgA* mutant through the extracellular polymerization of soluble CsgA subunits secreted from a *csgB* mutant.

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MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. MC4100 is a derivative of *E. coli* K-12 (14) and is a curli-producing strain ($CsgB^+$, $CsgA^+$). MHR204 is MC4100 *csgA2*::Tn*105* ($CsgB^+$, $CsgA^-$) (11), and MHR261 is MC4100 *csgB2* ($CsgB^-$, $CsgA^+$). The *csgB2* mutation has arisen through an insertion of 50 nucleotides derived from IS*10* Right and a duplication of 9 nucleotides of *csgB* DNA due to transposition of a Tn*104* element into the *csgB* gene followed by a nearly precise excision (11). MHR206 is MC4100 *csgD1*::Tn*105*; MHR203 is *csgE1*::Tn*104*; MHR334 is MC4100 *csgF1*::Tn*104*; and MHR210 is MC4100 *csgG1*::Tn*105* (11). *E. coli* strains DH5α and XL1-Blue have been described (15, 16). All experiments requiring expression of the *csg* genes were performed with cells grown on YESCA agar incubated at 28°C for 48 hr. YESCA agar is composed of 10 g/liter Casamino acids (Difco), 1 g/liter yeast extract (Difco), and 20 g/liter Bacto agar (Difco). Congo red indicator agar is YESCA agar containing 20 mg/liter Congo red (Sigma) and 10 mg/liter Coomassie brilliant blue G (Sigma) and was used to monitor the expression of curli in cells grown as colonies.

Construction of a *csgB* Transducing Phage. λMHR101 was constructed as follows. A plasmid was generated by filling in the ends of a CsgB-encoding *Hin*dIII/*Cla*I fragment from plasmid pcsg4 (9) and ligating this fragment to pRZ5202 linearized with *Sma*I. After transformation, a plasmid pMHR91 was isolated with the following order of markers: *bla-'csgD-csgDp-csgBAp-csgB-csgA'-lacZ*. This plasmid carries an operon fusion of a truncated *csgA* gene to the *lacZ* gene and, therefore, encodes the CsgB protein but not a functional CsgA protein. A phage stock was prepared by growing λRS45 on MC4100 containing pMHR91. λRS45 carries the genes *bla'-'lacZ* (17). Phages carrying *csgB* as a result of a double crossover event between homologous DNA in pMHR91 and λRS45 were identified by lysogenizing MHR261 and screening for blue colonies on medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside or screening for red colonies on medium containing Congo red. Pure $csgB^+$ phages (denoted λMHR101) were obtained by induction of purified positive lysogens. β-galactosidase expression in an MC4100 lysogen was determined as described (18) to confirm a pattern of expression similar to one found for *csgA* with Northern blot hybridizations (19).

Construction and Purification of a MalE–CsgA Fusion Protein. Plasmid pZB-aIII was constructed by PCR amplification of the *csgA* gene using the primers 5'-ATAGGAATTAATTCAGGTGTTGTTCC-3' and 5'-CGTATTCATA-

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AGCTTCTCCCGA-3′, ligation to pMAL-p2 (New England Biolabs) after digestion with *Hin*dIII and *Asp*700, and transformation into XL1-Blue.

Fusion protein was purified by affinity chromatography as follows. XL1-Blue/pZB-aIII grown in Luria broth was induced at an optical density of 0.6 with 0.3 mM isopropyl-thiogalactoside and further incubated for 3 hr. Cells were pelleted and periplasmic contents were released by osmotic shock treatment (20). After removal of spheroplasts by centrifugation, 100 ml of 5× column buffer (100 mM Tris·HCl, pH 7.4/1 M NaCl/5 mM EDTA/50 mM 2-mercaptoethanol/5 mM NaN_3) was added to 400 ml of supernatant (osmotic shock fluid). The osmotic shock fluid was applied to a column packed with amylose resin (New England Biolabs). After extensive washing, fusion protein was eluted with column buffer containing 20 mM maltose. Fractions containing the fusion protein were concentrated and desalted by passing over a Centricon concentrator (30-kDa cutoff; Amicon).

Preparation of an Anti-CsgA Antiserum. Rabbits were immunized with the purified MalE–CsgA fusion protein by Dakopatts (Glostrup, Denmark). Adsorption of the antiserum was performed as follows. Maltose binding protein (20 mg) purified from XL1-Blue/pMAL-p2 as described for the fusion protein was coupled to 10 ml of Sepharose 4B prepared according to the supplier's instructions (Pharmacia). The adsorbent was packed into a column and 10 ml of rabbit antiserum was passed through the column. The serum that did not adsorb was collected and extensively adsorbed against cell extracts of MHR265/pMAL-p2 (MHR265 is MC4100 containing a deletion in the *csg* locus rendering it $CsgA^-$) to further purify a CsgA-specific polyclonal antiserum: ZB-AIII. Despite extensive adsorbtion, ZB-AIII still contained contaminating antibodies against a few *E. coli* proteins.

Curli Production and CsgA Protein Analysis by Immunoblotting. Samples for SDS/PAGE were prepared by resuspending colonies of bacterial cells in SDS/PAGE sample buffer and boiling. This treatment does not depolymerize curli. When desired, depolymerization and solubilization of curli was achieved by resuspending cells in 90% formic acid followed by lyophilization before addition of SDS/PAGE sample buffer (7). Secreted CsgA protein was recovered from cells grown on agar plates by adding 10 ml of H_2O to the surface of the agar and leaving it at 4°C overnight to elute free proteins. The eluate was collected and separated from insoluble material by centrifugation for 1 hr at 140 000 × *g*. The supernatant was used for Western blotting without formic acid treatment.

After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell). Western blotting was performed using a 1:200 dilution of ZB-AIII in Tris-buffered saline and a secondary goat anti-rabbit antibody conjugated to alkaline phosphatase (Sigma).

Electron Microscopy. Electron microscopy was performed using a Philips CM 201 transmission electron microscope with copper grids (200-mesh; Sigma) coated with thin films of 2% Formvar. A suspension of bacteria in phosphate-buffered saline (PBS; 8 g/liter NaCl/1.44 g/liter Na_2HPO_4/0.24 g/liter KH_2PO_4, pH 7.4) was allowed to sediment for 2 min on a grid. After washing with PBS and blocking with 1% BSA/PBS, the grid was incubated with the ZB-AIII antiserum (1:200 dilution in 1% BSA/PBS) for 60 min at 37°C. After washes with PBS, the grid was incubated with protein A–gold (10-nm colloidal gold; Sigma) for 30 min at 37°C, rinsed with distilled water and negatively stained for 5 sec by 0.5% uranyl acetate.

Sequence Analysis. Sequence homology between the mature peptide of CsgA and the predicted mature peptide of CsgB was assessed using the program BESTFIT (Wisconsin Package, version 8, Genetics Computer Group) giving a quality score of 8 standard deviation units after randomization. The basic units of the repeat structures were defined using the program PRETTY (Wisconsin Package, version 8, Genetics Computer Group) using a plurality of 4. Secondary structures were predicted using criteria defined by Chou and Fasman (21).

Other Procedures. Standard immunoblotting and recombinant DNA procedures, and phage λ techniques were performed as described in refs. 22 and 23, respectively. The correct nucleotide sequences of all PCR-generated fragments and ligase-generated junctions were confirmed by sequencing.

RESULTS

Intercellular Complementation of a *csgA* Mutant by a *csgB* Mutant. We have previously isolated a pair of Congo red-negative mutants in *E. coli* K-12, neither of which can produce curli by itself. One mutant, MHR204, produces the CsgB protein but not the CsgA curli subunit protein (B^+A^-), whereas the other mutant, MHR261, produces CsgA but not CsgB (B^-A^+). Plating B^-A^+ and B^+A^- cells together results in Congo red-positive colonies, each of which is close to a negative colony. This Congo red-positive phenotype is seen only on a B^+A^- colony (recipient), grown close to a B^-A^+ colony (donor) as a differential staining by Congo red in the region facing the donor (Fig. 1*A*). A wild-type B^+A^+ cell on the other hand, is not an efficient donor for a B^+A^- cell, and a B^+A^- cell cannot act as a donor for a B^-A^+ cell (Fig. 1*A*). Intercellular curli formation can be viewed directly on single recipient cells with immunoelectron microscopy (Fig. 2). Thus, B^+A^- recipient cells (Fig. 2*C*) are seen to carry long fibers reacting with anti-CsgA antibodies (Fig. 2 *D* and *E*) when grown close to noncurliated B^-A^+ donor cells (Fig. 2*B*). The observed fibers are fewer and longer than those formed on B^+A^+ wild-type cells (Fig. 2*A*). The fact that the fibers are surface-bound indicates that the anchor for the fiber is located on the surface of B^+A^- cells.

Secretion and Polymerization of CsgA. The observed pattern of intercellular phenotypic complementation suggests that curlin is secreted in a soluble form and can be polymerized to a fibrous structure by a cell surface-bound protein either on the same cell or on adjacent cells. The monomeric and polymeric state of curlin can be examined by protein immunoblot analyses with anti-CsgA antibodies raised against a purified MalE–CsgA fusion protein. Curli polymers produced by B^+A^+ cells are not solubilized by boiling in SDS and can, therefore, be identified by antibodies to curlin as a fuzzy layer of insoluble material at the top of the stacking gel (Fig. 1*B*, lane 1). The polymer can be solubilized by treatment with formic acid upon which the curlin monomers enter the gel and migrate as a single 15-kDa band (Fig. 1*B*, lane 2). From Fig. 1*B*, it can be seen that B^-A^+ cells (lane 7) cannot form curli polymers but do contain some monomeric curlin. Fractionation experiments show that this protein originates from the periplasm (data not shown). Soluble monomeric curlin can also be recovered from the spent medium of a B^-A^+ donor strain and migrates in SDS/PAGE without any preceding formic acid treatment as a less defined band with an apparent molecular mass of 15 kDa (Fig. 1*C*, lane 2). Thus, in the absence of CsgB, the majority of the curlin monomers are secreted into the medium, where they remain as soluble monomers or possibly as oligomers sensitive to SDS. In the presence of CsgB provided by adjacent B^+A^- cells, SDS-insoluble polymers are formed on the B^+A^- recipient (Fig. 1*B*, lane 5). No soluble curlin is secreted into the medium from the wild-type B^+A^+ strain, explaining why it cannot act as a donor (Fig. 1*C*). Inactivation of the *csgG*, *csgFG*, or *csgEFG* genes or the whole *csgDEFG* operon abolishes curli production but results in neither a recipient (data not shown) nor a donor phenotype (Fig. 1*C*). The diffusion of curlin from the B^-A^+ donor, and hence curli formation, is significantly delayed by addition of an anti-CsgA serum to the agar, whereas the addition of an anti-MalE (maltose-binding protein) serum has no effect (data not shown). When a functional *csgB* gene is introduced into the

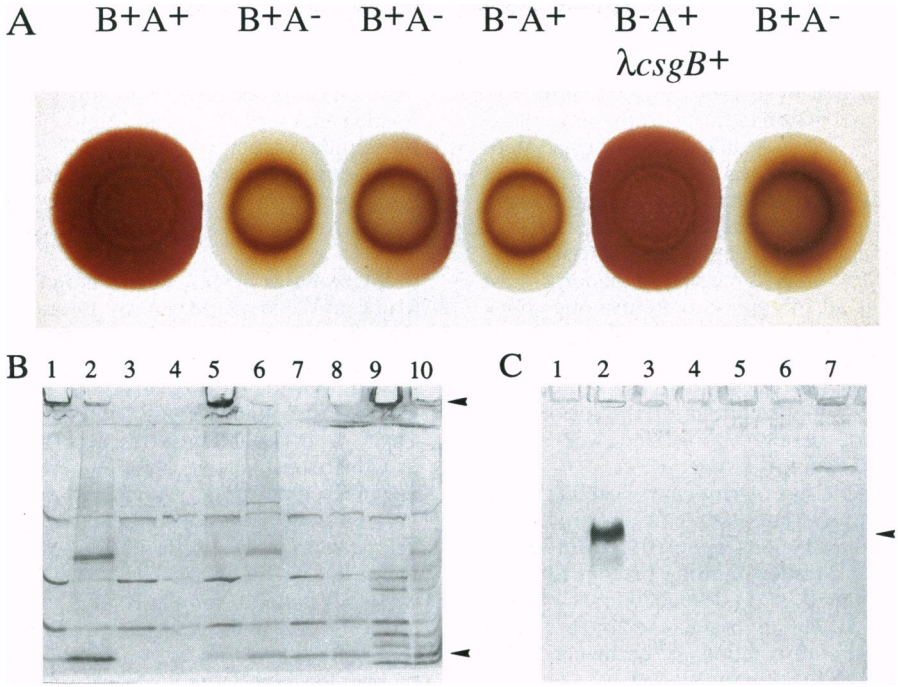

FIG. 1. Polymerization of soluble secreted CsgA protein (curlin). The symbols + or − show the status of expression of "B" protein (CsgB) and "A" protein (CsgA) from a particular strain. B^-A^+ λ*csgB*$^+$ denotes a *csgB* mutant genetically complemented with a functional, dominant allele of *csgB*. Such a strain is phenotypically B^+A^+. (*A*) Colonies grown on Congo red indicator agar from left to right: MC4100, MHR204, MHR204, MHR261, MHR261(λMHR101), MHR204. (*B*) Western blot of cell extracts from the *E. coli* colonies grown on the plate in *A* probed with an adsorbed polyclonal antiserum (ZB-AIII) raised against an MalE–CsgA fusion protein. Lane 1, MC4100 (B^+A^+); lane 2, same as lane 1 but treated with formic acid; lane 3, MHR204 (B^+A^-); lane 4, same as lane 3 but treated with formic acid; lane 5, MHR204 (recipient) grown in proximity to an B^-A^+ donor; lane 6, same as lane 5 but treated with formic acid; lane 7, MHR261 (B^-A^+, donor); lane 8, same as lane 7 but treated with formic acid; lane 9, MHR261(λMHR101) (*csgB*$^-$,csgA$^+$/λ*csgB*$^+$); lane 10, same as lane 9 but treated with formic acid. The λMHR101 carries the 5′ half of the *csgA* gene in addition to the *csgB* gene, giving rise to a truncated CsgA protein recognized by the antiserum. The arrows indicate the CsgA subunit monomer and the SDS-insoluble material at the top of the gel in lanes 1, 5, and 9 reacting with the CsgA antiserum, indicating polymeric CsgA protein. (*C*) Western blot with a CsgA-specific antiserum (ZB-AIII) used to probe proteins eluted from agar plates inoculated with MHR204 (lane 1), MHR261 (lane 2), MHR206 (lane 3), MHR203 (lane 4), MHR334 (lane 5), MHR210 (lane 6), or MC4100 (lane 7).

B^-A^+ mutant, it polymerizes its own curlin (Fig. 1 *A* and *B*, lane 9) and, as expected, no longer can act as a donor for the B^+A^- mutant (Fig. 1*A*).

DISCUSSION

Wild-type *E. coli* produces curli when deprived of nutrients at temperatures below 32°C in a low osmolarity medium. Both the CsgB and CsgA proteins, in addition to other gene products encoded by the *csgDEFG* operon, are necessary for formation of curli. However, curli production is restored in a *csgA* mutant when a *csgB* mutant is grown in close proximity. The *csgB* mutant does produce curlin but is unable to polymerize the subunits; instead, they are secreted into the medium in a soluble, polymerization-competent form. Upon the interaction with a cell-bound (nondiffusible) nucleator, curlin is polymerized into insoluble curli anchored to the *csgA* mutant cell.

The pathway leading to secretion of CsgA is not known. However, transposon insertions in any of the *csgE*, *csgF*, or *csgG* genes result in a nonsecretor phenotype, even though the same mutants have previously been shown to express CsgA (11). Since these transposon insertions are polar on the expression of downstream genes, we conclude that at least the distal gene in the operon, *csgG* (a putative lipoprotein), is required for the secretion of soluble CsgA into the medium.

We interpret the intercellular formation of curli as a self-assembly process. Provided that a nucleator is present, no other factors are needed to prime the polymerization of monomers. Once an initial interaction has occurred between the nucleator and monomer(s), the extension of the fiber is most likely achieved through addition of monomers to the free end. We find it likely that such an assembly pathway is not only true for the pair of mutants described here but also for the wild-type situation, independent of the source of secreted curlin. This is different from fimbriae biogenesis in Gram-negative bacteria where the filament is growing from the base through the addition of subunits from the periplasmic side of the outer membrane (24). Rather, formation of curli might have features in common with the assembly of the bacterial flagellum. This process depends on the self-assembly of a number of subunit proteins that will form the hook and the filament. The flagellin monomers are thought to be transported through a central channel in the filament for polymerization at the free end. The entire *Salmonella* flagellar filament can be reconstituted *in vitro* by sequential addition of monomeric FlaW, FlaU, flagellin, and FlaV proteins to preformed assemblies of hook protein (25) or to *flaV* mutant cells (26).

The fact that CsgB is homologous to CsgA suggests that CsgB could also be translocated over the inner and outer membrane and interact with CsgA in a manner related to the CsgA–CsgA interaction. N-terminal sequencing of CsgA purified from curli-producing cells shows that the protein is cleaved at a predicted signal peptidase processing site (12). Processing of CsgB at a predicted signal sequence would yield a 131-aa-long mature polypeptide, i.e., a protein of the same size as CsgA. When aligned for maximal similarity, the two mature polypeptides show 49% similarity and 30% identity. Inspection of the amino acid sequences of CsgB and CsgA

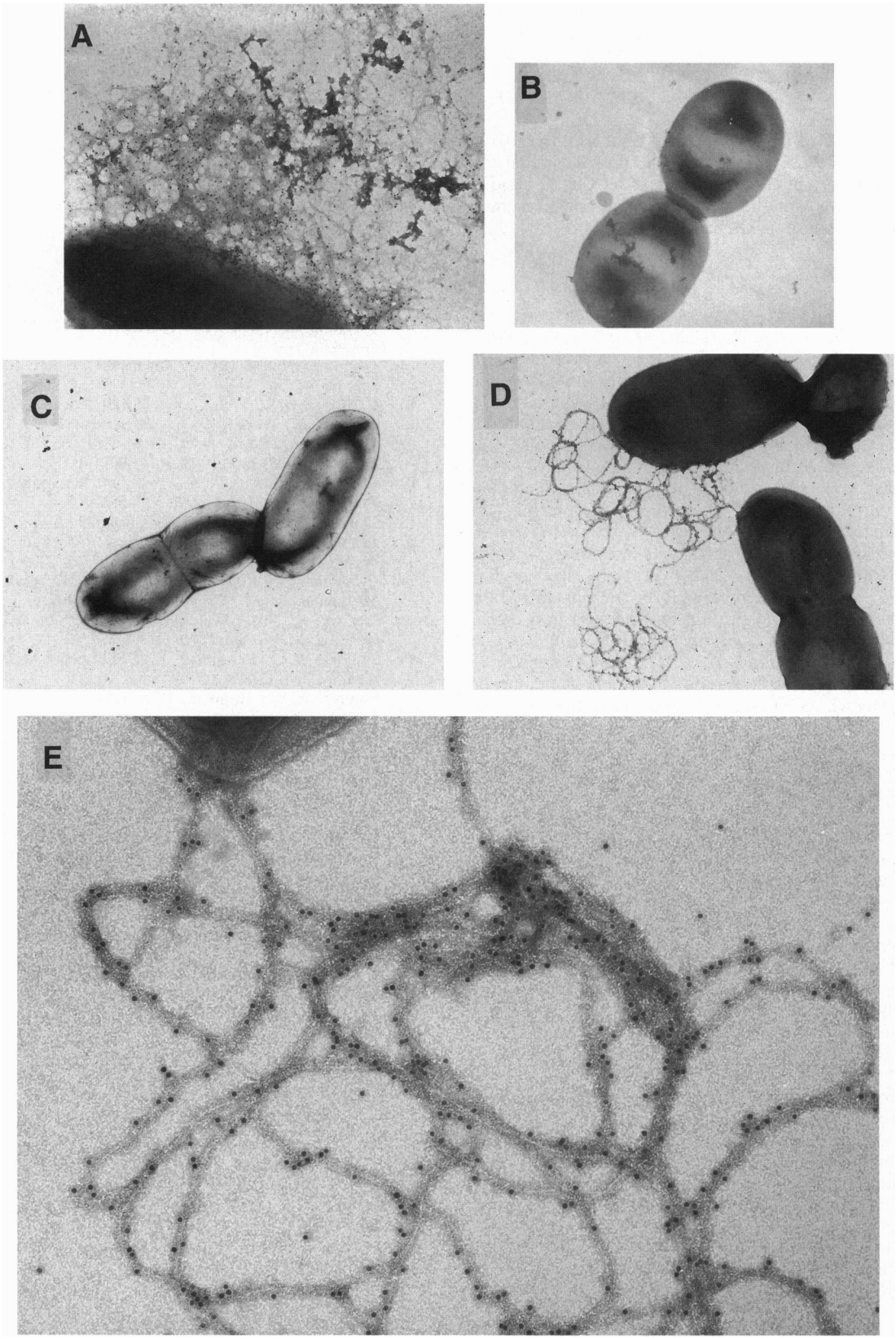

FIG. 2. Electron micrographs of negatively stained, ImmunoGold-labeled curli of *E. coli* cells from the colonies in Fig. 1. Curli polymers were labeled with protein A–gold after incubating with the ZB-AIII antiserum. (*A*) MC4100 (curliated, wild type); (*B*) MHR261 (B^-A^+); (*C*) MHR204 (B^+A^-); (*D* and *E*) MHR204 grown in proximity to the B^-A^+ donor. (*A*, ×18,000; *B*, ×9000; *C*, ×9000; *D*, ×9000; *E*, ×60,000.)

reveals the presence of a repeated motif. Computer-aided secondary structure predictions suggest that the repeats in the CsgA and CsgB amino acid sequences reflect a tertiary structure made up from a basic unit consisting of β-strand (7–8 aa)–turn (4 aa)–β-strand (7 aa)–turn (4 aa). The presence of five copies of the basic unit in the CsgA sequence can be visualized when aligned from residue Asn-22 (numbered from the N terminus of the mature protein):

```
.............NSELNIYQ YGGG NSALALQ TDAR
             NSDLTITQ HGGG NGADVGQ GSDD
             -SSIDLTQ RGFG NSATLDQ WNGK
             NSEMTVKQ FGGG NGAAVDQ TASN
             -SSVNVTQ VGFG NNATAHQ Y
```

CsgB is built up from a slightly different basic unit, occurring four times (or five if a less conserved unit at the N-terminal end is included) throughout the sequence and starting at residue Gln-25 (numbered from the N terminus of the presumed mature form of the protein):

```
.............QAAIIGQ AGTN NSAQLRQ GGSK
             LLAVVAQ EGSS NRAKIDQ TGDY
             NLAYIDQ AGSA NDASISQ GAYG
             NTAMIIQ KGSG NKANITQ Y
```

The first putative β-strand in the CsgA basic unit and the second putative β-strand in the CsgB basic unit is amphiphilic with alternating polar and apolar residues, suggesting the possibility that the major part of these proteins may fold as β-sheets connected by turns of four amino acids.

The simplest model for curli biogenesis would postulate that CsgB is the nucleator or part of a nucleator complex present on the cell surface. So far, we have not been able to demonstrate the presence of the CsgB protein by CsgB-specific antibodies. However, a MalE–CsgB hybrid protein is preferentially found in the outer membrane fraction using anti-MalE antibodies when the hybrid protein is expressed in a *csgBA* mutant. This mutant, expressing the hybrid protein, acts as a recipient in the presence of a *csgB* mutant, indicating a functional CsgB peptide associated with the outer membrane (unpublished data). As a putative nucleator at the base of the fiber, CsgB might be a very minor protein if only one molecule is required for each curli fiber.

We suggest that curli polymers are formed as a result of a conformational change of soluble CsgA initiated by an interaction with a nucleating CsgB protein. Such an induced conformational change might involve a conversion from a partially disordered structure in the monomeric state to readily ordered secondary structures in the polymeric state. This has been demonstrated in the self-assembly processes of the tobacco mosaic virus (27, 28) and the bacterial flagellar filament (29, 30). A similar change in conformation has been proposed to occur in the conversion from the cellular to the scrapie form of the prion protein (31, 32) that, like other amyloidogenic proteins, polymerizes into insoluble Congo red-binding fibrils (33) with extensive β-sheet structures.

Interestingly, curli-expressing cells bind Congo red, and a semipurified preparation of curli polymers viewed by polarization microscopy exhibits green birefringence typical for amyloid deposits (data not shown).

We believe that the extracellular nucleator-dependent biogenesis of adhesive curli organelles described here may provide a convenient model system to investigate fundamental principles governing primed polymerization of proteins.

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