BFA treatment, making them even easier to identify (Fig. 3, asterisks).

The partitioned matrix components were next tested for functionality. NRK cells that had entered metaphase in the presence of BFA were microinjected with a plasmid encoding the plasma membrane marker CD8 (28). After mitosis, BFA was washed out to permit repopulation of the Golgi matrix with enzyme-containing membranes. Two hours later, analysis showed that the re-formed Golgi mediated the transport of CD8 to the cell surface (Fig. 1C). As a control, co-injection of Sar1dn (29) prevented exit from the ER, even though the matrix components re-formed a ribbon-like structure.

As an alternative to BFA, we used Sar1dn to fractionate the Golgi apparatus. Synchronized NRK cells were injected with Sar1dn protein 5 to 6 hours before entry into mitosis, to trap the Golgi enzymes in the ER, leaving the matrix proteins behind. Matrix fragments containing GM130 partitioned at all stages of mitosis in a manner almost indistinguishable from that of untreated cells. Partitioning occurred in the absence of ManII, which was present throughout the ER (Fig. 4).

These two experimental methods for separating Golgi enzymes and Golgi matrix proteins emphasize a partitioning mechanism that is independent of the ER. This mechanism depends on Golgi matrix structures rather than the enzyme-containing membranes that normally populate them, which in turn suggests that these membranes are a less important part of the Golgi partitioning process. They could either travel with the matrix structures to the daughter cells (as we would argue) or get there via the ER (as would be argued by others). Thus, the two models are no longer mutually exclusive, and one could imagine that enzymes could take either or both routes. In this context, recent work on budding yeast suggests that the early Golgi is inherited via the ER, whereas the late Golgi is inherited autonomously (30). In the end, it may not matter how the enzymes are inherited, provided that there is accurate inheritance of the Golgi matrix (31).

References and Notes

14. Time-lapse phase images were acquired at 1-min intervals.
biofilm formation (1, 2) and mediate binding to a variety of host proteins (3–5).

Polymerized curli appear as 4- to 7-nm-wide fibers of varying lengths by negative-stain electron microscopy (EM) (6). Under high-resolution EM, curli appeared as a tangled and amorphous matrix surrounding the bacteria (Fig. 1A) (7). At higher magnifications, curli fibers appeared as ~6- to 12-nm-wide fibers of varying lengths (Fig. 1, B and C).

Curli were purified from MC4100 by sequential differential centrifugation (S6) and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (8). Resolution of CsgA, the major structural component of curli, required brief treatment with 90% formic acid (FA) to depolymerize the CsgA polymers into a ~17.5-kD protein and two minor proteins that migrated at ~30 and 32 kD (Fig. 1D). Only the 17.5- and 32-kD bands were unique to FA-treated samples, and these bands were recognized by antibodies to CsgA (anti-CsgA) (9). The migration of these proteins is consistent with monomer and dimer sizes of CsgA (10, 11). By EM, non-FA–treated S6 curli were indistinguishable from those presented naturally on the bacterial surface, appearing as aggregated fibers of varying lengths and widths (compare Fig. 1, E and F). Circular dichroism (CD) analysis indicated that these fibers were rich in β-sheet secondary structure with a minimum peak at ~218 nm (Fig. 2A).

Like other amyloid fibers, S6 curli induced a spectral change of a 10 μM Congo red (CR) solution with a maximum difference in absorbance between CR alone and CR bound to curli fibers at ~541 nm (Fig. 2B). Circular dichroism (CD) analysis indicated that these fibers were rich in β-sheet secondary structure with a minimum peak at ~218 nm (Fig. 2A).

Amyloid formation in eukaryotic cells is thought to be the result of a misguided protein-folding pathway. In contrast, E. coli possesses a specific nucleation-precipitation machinery encoded by the csgAB and csgDEFG operons to assemble curli. CsgB is thought to nucleate CsgA fibers (15). The csgDEFG genes encode CsgD, a FixJ-like transcriptional regulator, and three putative curli assembly factors, CsgE, CsgF, and CsgG. The lipoprotein CsgG localizes to the inner leaflet of the outer membrane and may serve as a curli assembly platform (16).

A nonpolar csgF– deletion mutant (MHR592) (17) resulted in aberrant CR binding properties. Wild-type bacteria stained CR positive after 30 hours of growth on YSCA plates (9). Strain MHR592 (csgF–) was CR negative after 30 hours of growth and only slightly CR-positive after 48 hours of growth (9). EM analysis of csgF– bacteria showed that fibers were less abundant but were otherwise indistinguishable from those produced by wild-type bacteria (Fig. 3A).

The monomeric and polymeric state of CsgA in the absence of CsgF was assessed. Very little SDS-soluble CsgA was present in extracts made from wild-type bacteria (Fig. 3B) because most of the CsgA subunits were assembled into curli as determined by the presence of a 17.5-kD band after pretreatment with FA (Fig. 3B). Similar to a csgB– mutant (Fig. 3B), most CsgA produced by a csgF– mutant remained in an SDS-soluble form after 48 hours of growth (Fig. 3B).

CsgA is secreted in a soluble, assembly-competent form by a csgB– mutant (CsgA+) donor and can be assembled on the surface of csgA– mutant (CsgB–+ recipient) bacteria in a process called interbacterial complementation (Fig. 3D) (18). CsgB+ recipient cells lacking the CsgA protein stained CR-positive when

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**Fig. 1.** High-resolution deep-etch EM micrographs of curliated E. coli and purification of curli fibers. (A and B) Representative freeze-fractured micrographs that have been rotary shadowed with platinum. The inset in (A) shows curli fibers. (C) MC4110 was absorbed onto glass and deep-etched without being fractured before rotary shadowing with platinum. (D) Coomassie stain SDS-PAGE of curli samples isolated from E. coli strain MC4100. Lanes 1 and 2 contain 40 μg of S6 wild-type curli without and with FA treatment, respectively. Lane 3 contains 20 μg of FA-treated GP curli. Molecular size markers (in kilodaltons) are indicated on the left. (E) Negative-stain EM micrographs of purified wild-type S6 curli. Bars: (A), 400 nm; (B) and (C), 60 nm; (E) and (F), 200 nm.
cross-streaked with either csgF- or csgF-B

double-mutant bacteria (Fig. 3D), indicating
that CsgA was secreted from csgF- cells and
assembled on the CsgB+ recipient cells. In
contrast, csgF- and csgF-B mutants were un-
able to accept CsgA from a CsgA+ -donating
strain (Fig. 3D). Thus, the curli assembly defect
in csgF- mutants was a nucleation defect
because CsgA produced by these cells was as-
sembly competent. CsgF may work independently
or in concert with CsgB to guide in vivo extra-
cellular nucleation of CsgA.

A nonpolar csgE- deletion mutant
(MHR480) (17) produced pale, non CR- bind-
ings colonies similar to those produced by a csgA
mutant (9). Despite the pale-colony phenotype,
MHR480 (csgE-), but not a csgE+/A- double
mutant, produced fibers that reacted with anti-
csgA. However, these structures were less
abundant than wild-type curli and were archi-
tecturally distinct in that they tended to arrange
into rings (Fig. 3C). In the csgE- cells, no
SDS-soluble CsgA could be detected, and the
total amount of SDS-insoluble CsgA was mark-
edly reduced (CsgA was detected in the FA-
treated sample only after extended exposure)
(Fig. 3B). A csgE- mutant was unable to donate
CsgA subunits when cross-streaked against the
CsgB+ recipient (Fig. 3D). However, a
csgE- mutant retained the ability to act as a
recipient and guide CsgA polymerization,
because it stained CR-positive when cross-
streaked against a CsgA+ donor (Fig. 3D). This
staining was weaker than that observed on a
CsgB+ recipient cross-streaked against a
CsgA+ donor (Fig. 3D), suggesting that in ad-
dition to the CsgA stability defect, csgE- bacte-
ria are also partially defective in their ability
to nucleate exogenous CsgA. A csgB- E- double
mutant was unable to accept or donate CsgA
(Fig. 3D).

To understand the requirements for subunit
polymerization, we purified CsgA in a soluble
form and analyzed its polymerization in vitro.
A six-histidine–tagged version of csgA (CsgA-
his) was cloned behind the IPTG (isopropyl-
B-d-thiogalactopyranoside)-inducible trc promot-
er in pHL3 (17), to create pMC3 (19). Plasmid-
derived CsgA-his complemented fiber forma-
tion and CR binding in a csgA- mutant (9). To
produce soluble, nonpolymerized CsgA, we
transformed pMC3 (csgA-his) into LSR6 (C600:
ΔcsgDEFG; ΔcsgBA) (19). However, at-
tempts to detect CsgA-his protein expressed in
LSR6 were unsuccessful (Fig. 4A). When
LSR6/pMC3 (csgA-his) was transformed with
pMC5 (csgEFG) or pMC1 (csgG), CsgA-his could
be detected in the culture supernatants
(Fig. 4A). Under these growth conditions (log-
arithmic growth in LB broth), only CsgG, and
not CsgE, was required for efficient CsgA sta-
bilization and secretion.

CsgA-his was purified from the superna-
pMC1 by standard nickel affinity chromatog-

Fig. 2. Amyloid-like properties of curli. (A) The CD spectrum of wild-type S6 curli was measured with 300 µg of protein in 10 mM tris (pH 7.4) with a 0.02-cm cell in a JASCO J715 spectropo-
larimeter at 25°C. S6 and GP curli gave similar CD spectra. (B) A 10-µM solution of CR prepared in 10 mM tris (pH 7.4) and 100 mM NaCl was filtered
through a 0.2-µm filter and mixed with 50 µL of buffer (10 mM tris (pH 7.4)) (●), S6 curli (4 mg/ml stock) (○), or GP curli (4 mg/ml stock) (×) in a final volume of 1 ml. All spectra were normal-
ized against the relevant non–CR-containing solutions. (C) Spectra representing the difference of CR with S6 curli and CR alone. (D) Fluorescence
of 5 µM ThT alone (○) or mixed with 40 µg of S6 curli (●) after excitation at 450 nm on an AlphaScan PTI fluorometer with a slit width of 4 nm. GP and S6 curli gave indistinguishable fluorescence results.

Fig. 3. Curli biogenesis in the absence of CsgE and CsgF. (A) Negative-stain EM micrographs of MHR592 (csgF-) bacteria grown on YESCA plates at 26°C for 48 hours. (B) CsgA visualized by Western analysis
with anti-CsgA (16) and bacteria grown at 26°C on YESCA plates for 48 hours. Circular plugs of 8 mm, including cells and underlying agar (to collect soluble, unpolymerized and secreted CsgA), were collected
and resuspended in 200 µL of 1.5× SDS loading buffer either with or without prior FA treatment. The extracts loaded in each lane are as follows: lanes 1 and 2, MC4100 (wild type); lanes 3 and 4, LSR10 (csgA-); lanes 5 and 6, MHR480 (csgE-); lanes 7 and 8, MHR261 (csgB-); and lanes 9 and 10 MHR592
(csgF-). (C) Negative-stain EM micrographs of MHR480 (csgF-) bacteria grown on YESCA plates at 26°C for 48 hours. These fibers often looped into imperfect circles (see inset). (D) Interbacterial complementation and CR binding in csgE- and csgF- mutants. The CsgA+ donor strain MHR261 (I) and the CsgB+ recipient strain LSR10 (II) were streaked from the top of the plate to the bottom. The horizontal cross-streaks were made from left to right with the following strains: MC4100 (wild type) (I-1 and II-1), csgA+ (I-2), csgB+ (II-2), csgE- (I-3 and II-3), csgEB+ (I-4 and II-4), csgF- (I-5 and II-5), and csgFB- (I-6 and II-6). Bars in (A) and (C), 200 nm.
phy (Fig. 4B). Immediately after elution from the nitriotrifluoracetic acid (NTA) agarose column, solutions containing purified CsgA-his were clear with no evidence of aggregation, and EM of this material revealed no fibers (9). CD analysis of this material indicated that soluble CsgA-his, unlike curli, was not rich in β-sheet secondary structure (Fig. 4C). However, after prolonged incubation (4°C for 4 to 12 hours), the CsgA-his solutions became opaque and noticeably viscous. EM analysis revealed that fibers had formed that were similar to those produced by wild-type bacteria (Fig. 4D), signifying that they had adopted the cross β structure conserved in all amyloid fibers. CsgA purified from cells expressing csgEFG or only csgG formed CR-binding fibers with indistinguishable kinetics (9). Thus, although CsgB and CsgEFG are required to facilitate efficient polymerization in vivo, they are not required for polymerization to proceed under these in vitro conditions. A critical function of the nucleation-precipitation assembly machinery may be to prevent CsgA polymerization within the cell and accelerate it at the cell surface.

Our demonstration that E. coli can produce extracellular amyloid-like fibers increases the recognized functional repertoire of amyloid fibers and provides a useful model system to study their formation. Purified amyloid fiber subunits associated with human diseases, such as the Aβ protein associated with Alzheimer’s disease, readily polymerize when incubated at high concentrations in vitro (20). However, the accessory proteins and conditions that facilitate in vivo polymerization of Aβ are incompletely understood. Understanding the regulation of curli subunit polymerization in E. coli will offer insight into the formation of eukaryotic amyloids. This work also raises the intriguing possibility that bacterial amyloids could play a role in certain human neurodegenerative and amyloid-related diseases. Future experiments will further examine the role of CsgB, CsgE, and CsgF during the in vivo polymerization of curli, and their function will be used as a model to understand the formation of other amyloids.

References and Notes
7. MC4100 was grown on YESCA plates at 26°C for 50 hours, washed with 1× phosphate-buffered saline, placed on anhydride-fixed slices of rabbit lung, and then quickly frozen by abruptly pressing the samples against a copper block cooled to 4 K with liquid helium. Frozen samples were fractured (where indicated), then deep-etched by exposure to a vacuum for 2.5 min, and replicas were made by rotary shadowing with a mixture of platinum and carbon (21). MC4100 was spread as a lawn on 10 150-mm YESCA plates and grown at 26°C for 60 hours before being scraped from the plates and suspended in 300 ml of 10 mM tris (pH 7.4) Buffer. The bacteria were pelleted by centrifuging at 9000 g for 10 min. The supernatant was mixed with an equal amount of 2× SDS-PAGE dye and heated to 95°C before gel electrophoresis. CsgA-his purified from cleared LSR6/pMC3/pMC5 supernatants filtered through a 0.2-µm filter before loading onto the gel (Fig. 4C). The gel-purified curli 2.5 µl of the 56 fraction was mixed with an equal amount of 2× SDS loading buffer and subjected to electrophoresis for 5 hours on a 12% SDS-PAGE gel. The curli remaining in the slot after electrophoresis were recovered as described (10).
21. MC4100 chromosomal DNA was amplified with the primers 5’-CATATGAAAC T T T TAAAAGTAGCAG-TAT T TACGCTG and 5’-GGATCCTCAGGAT TCCGGTG-GGATCCTCAGGAT TCCGGTG, and the amplicon was cloned into the Nde I and Eco RI sites of pHL3 to create pMC2. This work also raises the intriguing possibility that bacterial amyloids could play a role in certain human neurodegenerative and amyloid-related diseases. Future experiments will further examine the role of CsgB, CsgE, and CsgF during the in vivo polymerization of curli, and their function will be used as a model to understand the formation of other amyloids.
At most bacterial promoters, RNA polymerase (RNAP) holoenzyme (σββ′σ′αα′′) recognizes sequence elements centered ~10 and ~35 nucleotides upstream of the initiation point, with the σ subunit specifically contacting both promoter elements [reviewed in (7)]. Different sigma share four evolutionarily conserved regions, which can be further subdivided (1). Centrally located region 2.4 interacts with the −10 promoter element, and COOH-terminal region 4.2 interacts with the −35 element (1). Because most free σ subunits cannot recognize promoters, conformational changes in core RNAP, σ, or both must occur during holoenzyme formation. Indeed, luminescence resonance energy transfer (LRET) measurements show that the Escherichia coli RNAP core induces a change in σ70, the principal σ (2). As a result, the distance between σ70 regions 2.4 and 4.2 increases dramatically, to match the distance between the promoter elements (2). The mechanism by which the conformation of σ is altered upon holoenzyme formation has not been defined, nor have the core interaction sites that bring about this change been identified.

A structure of core RNAP from eubacterium Thermus aquaticus has been determined (3). One structural element, the “flexible flap” (comprising conserved segment G of the RNAP β subunit), protrudes away from the body of the enzyme (Fig. 1). An E. coli RNAP mutant lacking β amino acids 900 through 909 at the tip of the flap was previously found to be defective in transcription initiation unless the initiation region was pre-melted (4). To further examine this defect, we deleted the entire flap from E. coli RNAP (5). Inspection suggests that the RNAP structure should be minimally perturbed by the deletion (Fig. 1).

Mutant RNAP was purified (6), and the ability of mutant holoenzyme (Er70) to initiate transcription from T7 A2, a strong −10/−35 promoter, was tested (7). Wild-type Er70 was active at T7 A2; in contrast, mutant Er70 was inactive (Fig. 2A). Transcription from the gal/P1 promoter was also tested. This promoter belongs to a class of promoters whose −10 elements are extended by an upstream dinucleotide TG (8). σ region 4.2 is not required for recognition of extended −10 promoters, due to additional RNAP contacts with the TG motif (8). Er70 lacking the β-flap was active at gal/P1 (Fig. 2A). These results suggest that the β-flap is important for transcription from −10/−35 promoters, but is dispensable for transcription from extended −10 promoters.

Wild-type Er70 protected T7 A2 promoter DNA from deoxyribonuclease I (DNase I) digestion (Fig. 2B) (7). In contrast, the pattern of DNase I digestion in reactions containing mutant Er70 was similar to the naked DNA pattern, suggesting that Er70 lacking the β-flap is unable to form complexes with −10/−35 promoters.

The restricted promoter specificity caused by the β-flap deletion could be direct (i.e., the flap contributes directly to promoter recognition) or indirect (i.e., the flap positions σ region 4.2 for interaction with the −35 element). The following experiments support the second possibility. We studied σ70 region 4.2-DNA interactions in gal/P1 complexes, where region 4.2 makes favorable, but nonessential DNA interactions ~35 base pairs (bp) upstream of the initiation point (8). Overall, the gal/P1 complexes formed by mutant Er70 appeared similar to the wild-type complexes (Fig. 2C) (8). However, DNA between positions −3 and −39 was protected in the wild-type, but not in the mutant complexes (Fig. 2C, arrowheads), suggesting that in the absence of the β-flap, interactions between σ and gal/P1 upstream DNA do not occur.

To show directly that the β-flap is required for the conformational change in σ that occurs upon holoenzyme formation, we used LRET, which uses energy transfer between a luminescent donor and fluorescent acceptor to determine atomic-scale distances between the probes (9). LRET donor and acceptor probes were incorporated into different σ domains, and inter-