Bactofilins are a recently discovered class of cytoskeletal proteins of which no atomic-resolution structure has been reported thus far. The bacterial cytoskeleton plays an essential role in a wide range of processes, including morphogenesis, cell division, and motility. Among the cytoskeletal proteins, the bactofilins are bacteria-specific and do not have a eukaryotic counterpart. The bactofilin BacA of the species Caulobacter crescentus is not amenable to study by x-ray crystallography or solution nuclear magnetic resonance (NMR) because of its inherent noncrystallinity and insolubility. We present the atomic structure of BacA calculated from solid-state NMR—derived distance restraints. We show that the core domain of BacA forms a right-handed \( \beta \) helix with six windings and a triangular hydrophobic core. The BacA structure was determined to 1.0 \( \AA \) precision (heavy-atom root mean square deviation) on the basis of unambiguous restraints derived from four-dimensional (4D) HN-HN and 2D C-C NMR spectra.

The cytoskeleton was once thought to be a unique feature of eukaryotic cells, but homologs to all major components of the eukaryotic cytoskeleton have also been found in prokaryotes (1–3). Additionally, bacteria-specific cytoskeletal elements have been identified. Among them are the bactofilins, a recently discovered family of cytoskeletal proteins that occur in most bacterial lineages (4–6). Bactofilins have a central conserved domain and variable proline-rich flanking terminal regions. They polymerize spontaneously and independently of nucleotides or other cofactors and are involved in a variety of crucial cellular processes. In the prosthecate \( \alpha \)-proteobacterium Caulobacter crescentus, the two bactofilin paralogs BacA and BacB assemble into membrane-associated polymeric sheets that are specifically localized to the cell pole carrying the stalk—a thin protrusion of the cell body involved in cell attachment and nutrient acquisition (4). These assemblies serve as spatial landmarks mediating the polar localization of the cell wall biosynthetic enzyme PbpC, which is involved in stalk biosynthesis and organization. The human pathogen Helicobacter pylori, by contrast, requires the bactofilin homolog CcmA for maintaining its characteristic helical cell shape, a feature required for cells to efficiently colonize the gastric mucus (7).

Recently, we demonstrated that high-quality solid-state nuclear magnetic resonance (ssNMR) spectra of BacA could be obtained, which allowed us to achieve the resonance assignment for the central core domain (residues 37 to 139) (8). As determined from conformation-dependent chemical shifts (9), BacA adopts a structure with a rigid core comprising 18 \( \beta \) strands and flexible N- and C-terminal regions. On the basis of the assigned \( \beta \) strand segments and additional data on the mass per length of the BacA filaments from scanning transmission electron microscopy, a \( \beta \)-helical fold was proposed for the BacA subunit (8). Additionally, homology modeling of BacA (8) and its Myxococcus xanthus homolog BacM (10) suggested a left-handed \( \beta \)-helical arrangement. Because no cytoskeletal filament protein has yet been reported to adopt a \( \beta \)-helical fold, the determination of an atomic-resolution structure of a member of the bactofilin family was highly desirable.

X-ray crystallography and solution NMR are difficult to apply to bactofilins because of the rapid polymerization of the subunits into fibrillar structures during overproduction. In light of the high-quality ssNMR data reported so far (8) and the recent success in using ssNMR to solve the atomic structures of supramolecular protein assemblies (11–13), we therefore set out to determine the structure of the BacA subunit by ssNMR.

First, we analyzed spectra of sparsely \( ^{13} \)C-labeled BacA samples obtained by growing BacA-overproducing Escherichia coli cells in media that contained either \([1,3^{-13} \text{C}] \) glycerol or \([2^{-13} \text{C}] \) glycerol as the sole carbon source (14, 15). The resulting samples enabled the acquisition of carbon-carbon correlation spectra of excellent quality and resolution (see figs. S1 and S2). The \([2^{-13} \text{C}] \) glycerol–labeled BacA sample yielded spectra containing many cross peaks in the aromatic and \( \alpha \) regions (fig. S1) that could be unambiguously assigned and converted into long-range (that is, \( |i − j| > 4 \) ) distance restraints. Similarly, spectra from the \([1,3^{-13} \text{C}] \) glycerol; \([U^{-15} \text{N}] \) labeled BacA sample provided long-range distance restraints between side-chain methyl groups (fig. S2). On the basis of a total of 374 reliable \( ^{13} \text{C}^{-13} \text{C} \) long-range distances and additional 172 torsion angle restraints, predicted from the assigned backbone atom chemical shifts (table S1) by the software TALOS-N (16), a \( \beta \) helical structure with six windings was calculated. Although right-handed models were more abundantly generated by the structure calculations using Xplor-NIH (17), both left-handed (1 to 2 of 20) and right-handed (18 to 19 of 20) \( \beta \) helices were among the 10% (20 of 200) of the lowest-energy structures.

To unambiguously identify the handedness of the \( \beta \) helix, we collected short [that is, \( d[H,H] \leq 5 \AA \) proton-proton distance restraints from four-dimensional (4D) HNN(H)(H)NH (18, 19) and 2D NHHC (20) spectra. The excellent resolution of the cross peaks in the proton-detected \( ^{1} \text{H}^{-15} \text{N} \) correlation NMR spectrum (Fig. 1A) of deuterated and 100% back-exchanged BacA37–139 allowed the unambiguous identification of HN-HN distance restraints from a 4D spectrum. Initially, a total of 96 amide proton resonances were assigned using a
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series of 3D proton-detected NMR experiments (21). The 4D HN(H)NH experiment used 5.2-ms radio frequency–driven recoupling (RFDR) mixing (22) for the magnetization transfer between adjacent NH pairs (fig. S3). To reduce the measurement time of the 4D experiment, the data were acquired by sine-weighted Poisson-gap nonuniform sampling (NUS) of 25% of the total number of data points (23, 24). In the resulting spectrum, the chemical shifts of HN, N, N, and HN were correlated on the basis of the HN-HN dipolar interaction. For example, in the 2D plane defined by the HN and N chemical shifts of residue L73, cross peaks were exclusively observed to residues spatially close to L73 (Fig. 1, B and C). The distance restraints collected by this method agreed well with the β-helix model generated by our earlier calculations based on carbon-carbon distances and predicted dihedral angles (see above). Because the peaks are very well separated in the 4D spectrum, this approach significantly reduces assignment ambiguities. A total of 59 unambiguous HN-HN distance correlations (present in both of the corresponding HN correlation planes) were extracted (table S2). Structure calculations (see below) showed that these restraints fitted only to the model of the right-handed β helix. These results could be further corroborated by HN-H contacts extracted from an NHHC spectrum recorded on the [1,3-13C]-glycerol; U-15N]-labeled BacA sample (fig. S4).

In total, 1932 distance restraints were collected, including 178 medium-range (that is, |i − j| = 2, 3, or 4) and 488 long-range contacts (fig. S5). These peaks were selected in an iterative manner. Before the first structure calculation, only unambiguous peaks were selected. The resulting structure was then used to select additional (structurally unambiguous) peaks for refinement. This was repeated in several rounds until almost all of the peaks in the spectra were assigned. Additionally, the 172 torsion angle restraints predicted by TALOS-N and an additional 116 β sheet hydrogen bond restraints were used in the NMR structure calculation of the rigid central domain (residues 37 to 139), resulting in the structure depicted in Fig. 2. The heavy-atom average root mean square deviation from the mean structure of the 20 lowest-energy conformers out of 200 calculated structures is 0.4 Å for the backbone and 1.0 Å for all heavy atoms, indicating a high precision of the calculated structure ensemble.

The overall organization of the BacA core domain is a right-handed β helix with six windings and a triangular hydrophobic core. The core is defined by three β strands per winding that form continuous parallel β sheets (Fig. 2C). Many of the “corners” of the β helix are formed by glycines, a feature that is commonly seen in β-helical structures. These glycines are highly conserved within the bactofilin family (8). The β sheet arrangement is stabilized by hydrogen bonds between adjacent strands and hydrophobic side chains tightly packed in the interior of the β helix. All charged residues face toward the outside, and some of them are arranged on top of each other such that their charges mutually compensate each other (E49-K65-E82, E57-R72, E88-K103-E120, and D116-R133). The first (#1) and the last (#6) windings of the β helix are not as well defined by the data as windings #2 to #5. Here, we could not detect any intermolecular distance restraints between windings #1 and #6 as would be expected from a head-to-tail arrangement of the subunits. The lack of such restraints could either indicate a different supramolecular arrangement or reflect an increased local mobility of windings #1 and #6. All of our observed distance restraints were unambiguously assigned to intrasubunit contacts, leaving no unassigned restraints that could have arisen from intermolecular subunit-subunit contacts. This suggests that the lack of restraints for windings #1 and

Fig. 1. Structural restraints from 1H-detected ssNMR. (A) 2D H-N correlation spectrum of deuterated and 100% back-exchanged BacA37–139 with some of the amino acid residues annotated. (B) One plane of the 4D HN(H)NH correlation spectrum. Plane taken at chemical shift positions of the L73 residue (diagonal peak marked in red). Long-distance restraints (cross peaks) annotated in black. For better visualization, the corresponding residues are labeled in blue in (A). The appearing cross peaks represent spatially close amide protons and are used for structure calculation. (C) Schematic representation of magnetization transfer from residue L73 to spatially close residues during the 4D NMR experiment (blue arrows). Dashed red lines represent hydrogen bonds between consecutive windings of the β helix.

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#6 results from increased dynamic motion in these regions. This possibility is further supported by the observation that cross-peak intensities in the dipolar-based correlation spectra used for the assignment were generally attenuated for residues located within these windings (Fig. S6). Notably, mutations in winding #6 (L122S, M124A, M124S, and F130A) were previously shown to affect BacA assembly in vivo, whereas an A123S exchange did not. On the basis of the arrangement of winding #6 determined here, the side chains of L122, M124, and F130 point to the hydrophobic core, whereas that of A123 points outside (see Fig. 2C). Mutation of the three core residues may thus destabilize winding #6 or render the surface provided by winding #6 less hydrophobic, thereby potentially preventing intersubunit interaction.

Here, we have determined a de novo atomic-resolution structure of the bactofilin BacA, on the basis of a large number of unambiguous ssNMR-derived distance restraints. Thus far, the right-handed amyloid state of the BacA structure is similar to that of the prion protein HET-s in its fold has not been described for any other cytoskeletal protein. However, windings #5 or render the surface provided by winding #6 less hydrophobic.

Fig. 2. Structure of the BacA subunit (residues 37 to 139). (A and B) Top view (A) and side view (B) of BacA. (C) Schematic representation of the six windings. Hydrophobic residues are colored white, acidic residues are colored red, basic residues are colored blue, and others are colored green. Mutations of residues L122, M124, and F130 (winding #6) to alanine or serine affect the BacA assembly in vivo. These residues are marked with an asterisk.

#6 results from increased dynamic motion in these regions. This possibility is further supported by the observation that cross-peak intensities in the dipolar-based correlation spectra used for the assignment were generally attenuated for residues located within these windings (Fig. S6). Notably, mutations in winding #6 (L122S, M124A, M124S, and F130A) were previously shown to affect BacA assembly in vivo, whereas an A123S exchange did not. On the basis of the arrangement of winding #6 determined here, the side chains of L122, M124, and F130 point to the hydrophobic core, whereas that of A123 points outside (see Fig. 2C). Mutation of the three core residues may thus destabilize winding #6 or render the surface provided by winding #6 less hydrophobic, thereby potentially preventing intersubunit interaction.

Here, we have determined a de novo atomic-resolution structure of the bactofilin BacA, on the basis of a large number of unambiguous ssNMR-derived distance restraints. Thus far, the right-handed β-helical fold has not been described for any other cytoskeletal protein. However, the BacA structure is similar to that of the prion protein HET-s in its amyloid state. It will be interesting in the future to investigate the arrangement of BacA in the context of the membrane-associated polymeric sheets observed in vivo by complementary techniques such as cryo-electron microscopy and tomography to determine a hybrid structure of the whole assembly.

MATERIALS AND METHODS

Sample preparation
To prepare 13C-labeled samples, we produced BacA-His6 in M9 medium with 0.3% (v/v) [2-13C]glycerol or [1,3-13C]glycerol as the sole carbon source. The [1,3-13C]glycerol-containing medium additionally contained 15NH4Cl as the sole nitrogen source. The overproduction protocol was based on a previously described procedure. Briefly, cells of E. coli Rosetta(DE3)pLysS (Invitrogen) were transformed with pMT879 and grown in 1 liter of SB medium supplemented with appropriate antibiotics at 37°C to an OD600 (optical density at 600 nm) of 3 to 4. Cells were harvested by centrifugation and washed with 100 ml of M9 salt solution. The pellets were then resuspended in 1 liter of M9 medium containing the indicated carbon and nitrogen sources. The cultures were grown for 1.5 hours at 37°C. Subsequently, the production of BacA-His6 was induced by the addition of 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG). After 24 hours of cultivation, the cells were harvested, and BacA-His6 was purified as described previously. The samples, together with a few crystals of DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid), were packed into 4-mm magic angle spinning (MAS) rotors, using protein quantities of ~30 mg each.

To produce a shortened version of BacA that corresponded to the rigid DUF583 domain of BacA, we cloned the coding sequence for amino acids 37 to 139 into a modified pET28a vector (Clontech), encoding a cleavable N-terminal heptahistidine tag. After transformation into E. coli BL21(DE3), cells were grown at 37°C in an M9 minimal medium with 15NH4Cl and 13C6-D-glucose as sole nitrogen and carbon sources, respectively, prepared in 99.9% D2O. Overproduction of the [3H,13C,15N]-labeled fusion protein was induced by the addition of 0.5 mM IPTG. The culture was harvested 15 hours after induction, resuspended in lysis buffer [50 mM tris-HCl (pH 8.0), 500 mM NaCl, 5 mM imidazole, and 0.5 mM phenylmethylsulfonyl fluoride] supplemented with Benzonase (25 U/ml; Merck Millipore), and lysed by sonication. From the supernatant, the fusion protein was purified by
immobilized metal affinity chromatography on Ni-NTA (nickel-nitrilotriacetic acid) Sepharose. The protein was eluted with 20 column volumes of buffer with 300 mM imidazole. The eluate was dialyzed against tobacco etch virus (TEV) cleavage buffer [50 mM tris-HCl (pH 8.0), 0.5 mM EDTA, and 1 mM dithiothreitol], and the fusion tag was cleaved with 1 mg of TEV per 100 mg of fusion protein overnight at room temperature before removal with Ni-NTA Sepharose. The concentration of the protein was adjusted to 1.5 mg/mL, and the sample was kept at room temperature for 8 days. The resulting aggregate, formed from a total of 42 mg of protein, was collected by centrifugation. The sample was transferred into a 1.9-mm NMR rotor equipped with a bottom spacer. Before closing the rotor, a few crystals of DSS and about 1 µl of D2O were added.

**NMR spectroscopy and detection of distance restraints**
Carbon-detected ssNMR experiments were conducted on 16.4- and 18.8-T (1H resonance frequencies of 700 and 800 MHz, respectively) wide-bore NMR spectrometers (Bruker Biospin) equipped with 4-mm triple-resonance (1H, 13C, and 15N) MAS probes. Spectra were recorded at an MAS rate of 11 kHz and were calibrated with DSS as an internal proton chemical shift reference. The sample temperature was kept constant at about 4°C, as measured by the temperature-dependent position of the water resonance peak with respect to internal DSS (30). High-power 1H, 13C decoupling with a radio-frequency amplitude of about 80 kHz was applied during evolution and detection periods (31).

To detect medium- and long-range contacts, we used the proton-driven spin diffusion (PDSD) scheme with a longitudinal mixing time of 800 ms for BacA produced with [1-13C]glycerol and mixing times of 300 and 700 ms for BacA produced with [1,3-13C2]glycerol. An NHHC (20) spectrum with a (1H,1H) mixing time of 200 µs was recorded at 18.8 T using BacA produced with [1,3-13C2]glycerol. Contact times of tNH = 300 µs and tHC = 90 µs were used to ensure polarization transfer within NH or CH2 (x = 1, 2, or 3) groups only.

The 1H-detected NMR spectra were acquired on a Bruker 21.2-T (900-MHz 1H Larmor frequency) standard-bore NMR magnet equipped with a 1.9-mm, four-channel (1H, 13C, 15N) probe (32). The MAS frequency was set to 40 kHz, and the sample temperature was set to 28°C. The field was locked to the deuterium resonance resulting from the added D2O.

Backbone chemical shift assignment was achieved using five 3D NMR spectra: (H)CANH, (H)CONH, (H)CACO(N)H, (H)CÖCA(N)H, and (H)CA(CO)NH. The used assignment strategy and experimental details closely follow the protocols described earlier (21, 33, 34). The long-distance restraints were collected using a 4D NMR [HN(H)(H)NH] spectrum with RFDR mixing (22) for the magnetization transfer between spatially adjacent NH pairs. RFDR inversion pulses with a radio-frequency amplitude of 100 kHz were applied. The RFDR mixing time was 5.2 ms. 1H, 13C, and 15N chemical shifts are listed in table S1 and were deposited into the Biological Magnetic Resonance Data Bank (BMRB entry: 25642). An overview of the experimental details is provided in table S3.

To reduce the measurement time of the 4D NMR spectrum from ~36 to ~9 days, the data were acquired by sine-weighted Poisson-gap NUS of 25% of the data points (23, 24). The reconstruction of the missing free induction decays (FIDs) was carried out with the iterative soft-threshold method implemented in the hmsIST software. Spectral processing was carried out with NMRRipe 8.2 (35). A squared sine bell window function with a shift of π/3 was applied to the 512 acquired complex points of the FIDs. After zero-filling to 1024 complex points, the FIDs were Fourier-transformed and the region between 10 and 5 parts per million (ppm) was extracted. The three indirect dimensions were then reconstructed by hmsIST to 64 complex points for each dimension, with 3000 iterations and a level multiplier of 0.995. A squared sine bell window function with a shift of 2π/3 was applied to each indirect dimension. Subsequent Fourier transform was carried out after zero-filling to 128 complex points for each indirect dimension. A phase correction was only applied for the direct dimension. The resulting NMR spectrum was viewed and assigned in CcpNmr Analysis version 2.4 (36). Chemical shift referencing was carried out by overlaying the referenced 2D H-N correlation spectrum.

**Structure calculation and validation**
Backbone φ and ψ torsion angle restraints were predicted from the patterns of the backbone atom chemical shifts using the TALOS-N software package. Assigned ssNMR cross peaks from the PDSD spectra were manually converted into internuclear carbon-carbon distance restraints with a single distance range of 1 to 8 Å. The information extracted from NHHC and 4D HN(H)(H)NH spectra was converted into interproton distance restraints with a distance range of 1 to 6 Å (fig. S7). Hydrogen bond donor and acceptor atoms used for the final structure refinement were identified on the basis of the secondary structure elements predicted by TALOS-N and by manual inspection of earlier rounds of structures.

All structure calculations were performed using Xplor-NIH version 2.37. With an extended template structure, calculations were initiated by randomizing the backbone torsion angles and then running 50 cycles of Powell energy minimization to remove close nonbonded contacts. Simulated annealing was performed in two stages, using 20,000 steps at 1500 K and 30,000 steps with gradual cooling to 100 K using a constant time step of 5 fs. Finally, the annealed structures were energy-minimized using a 200-step Powell energy minimization. The 20 lowest-energy structures out of 200 calculated structures were selected.

Visualization was performed using PyMOL (37). Atomic coordinates and structure constraints were deposited in the Protein Data Bank (PDB ID: 2N3D). The quality of the final ensemble of 20 NMR structures was evaluated by submission to the Protein Structure Validation Software suite version 1.5 (38). A summary of the restraints used in the final structure calculations and the global structure quality factors is provided in table S4.

**SUPPLEMENTARY MATERIALS**
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/1/11/e1501087/DC1
Fig. S1. 2D 13C-13C PDSD spectrum of [2-13C]glycerol-labeled BacA.
Fig. S2. 2D 13C-13C PDSD spectrum of [1,3-13C2]glycerol-labeled BacA.
Fig. S3. Schematic representation of the 4D HN(H)(H)NH sequence.
Fig. S4. 2D NHHC spectrum of [1,3-13C2]glycerol-labeled BacA.
Fig. S5. BacA sequence and distance restraints used for structure calculation.
Fig. S6. Intensities of the cross peaks in the 3D H/H/C cross spectrum.
Fig. S7. Schematic representation of the approximate amide proton–amide proton distances within the parallel β sheets.
Table S1. Assigned chemical shifts of BacA (in ppm, relative to internal DSS).
Table S2. Summary of the distance restraints collected from the 4D HN(H)(H)NH spectrum.
Table S3. Experimental details.
Table S4. BacA ssNMR structure calculation statistics.
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The BacA chemical shifts were deposited in the BMRB under accession code 25642, and its structure the Supplementary Materials. Additional data related to this paper may be requested from the authors. The BacA chemical shifts were deposited in the BMRB under accession code 25642, and its structure the Supplementary Materials. Additional data related to this paper may be requested from the authors. The BacA chemical shifts were deposited in the BMRB under accession code 25642, and its structure the Supplementary Materials. Additional data related to this paper may be requested from the authors.