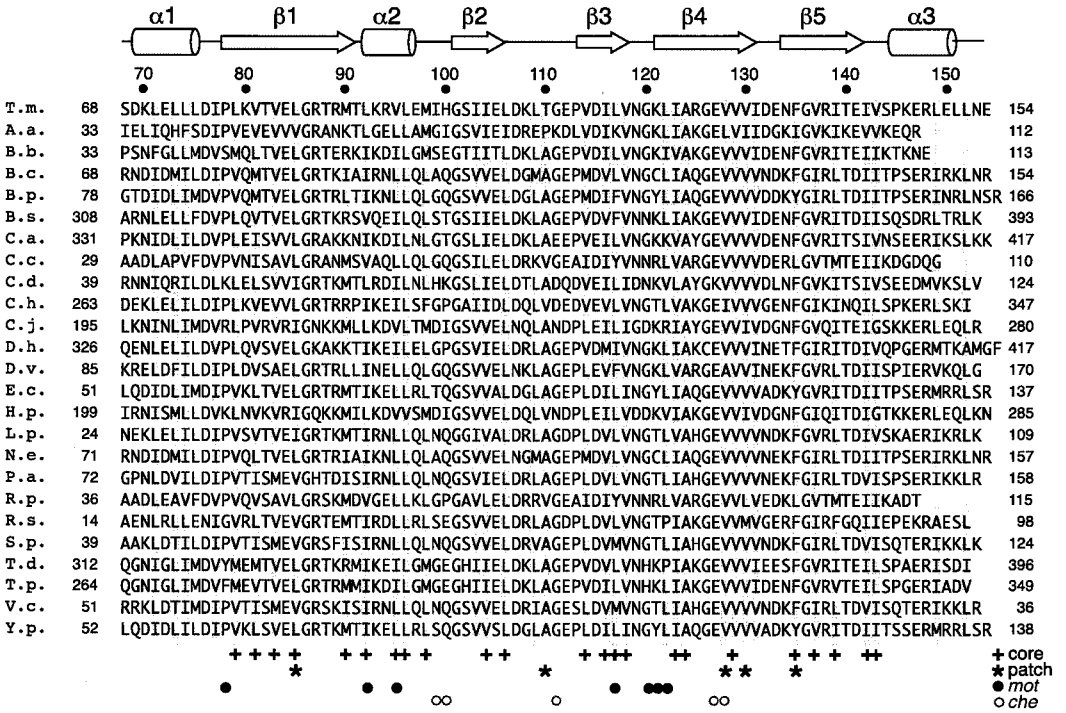

FIG. 4. FliN sequence alignment for the part of FliN whose structure is reported here (residues 68 to the end for the *T. maritima* protein). The N-terminal part of the protein is more variable and is partially dispensable for function in *E. coli* (65). The secondary structure is indicated at the top. Gray bars in the alignment indicate positions where the hydrophobic character is conserved. At the bottom plus signs indicate residues that are mostly buried and contribute to the core of the FliN dimer, asterisks indicate residues that make major contributions to the surface hydrophobic patch, solid circles indicate the positions of mutations that give an immotile but flagellate phenotype, and open circles indicate the positions of mutations that give a motile but nonchemotactic (switching-impaired) phenotype. T.m., *Thermotoga maritima*; A.a., *Aquifex aeolicus*; B.b., *Borrelia burgdorferi*; B.c., *Burkholderia cepacia*; B.p., *Bordetella pertussis*; B.s., *Bacillus subtilis*; C.a., *Clostridium acetobutylicum*; C.c., *Caulobacter crescentus*; C.d., *Clostridium difficile*; C.h., *Carboxydothermus hydrogenoformans*; C.j., *Campylobacter jejuni*; D.h., *Desulfitobacterium hafniense*; D.v., *Desulfovibrio vulgaris*; E.c., *Escherichia coli*; H.p., *Helicobacter pylori*; L.p., *Legionella pneumophila*; N.e., *Nitrosomonas europaea*; P.a., *Pseudomonas aeruginosa*; R.p., *Rhodopseudomonas palustris*; R.s., *Rhodobacter sphaeroides*; S.p., *Shewenella putrefaciens*; T.d., *Treponema denticola*; T.p., *Treponema pallidum*; V.c., *Vibrio cholerae*; Y.p., *Yersinia pestis*.

indicate a specific functional role for this part of the protein surface.

Association state of FliN and a stable FliM-FliN complex. The crystal structure shows that FliN is likely to be a dimer or a larger molecule in solution. To study the association state of FliN in solution, we performed analytical ultracentrifugation experiments with purified FliN proteins from both *T. maritima* and *E. coli*. *T. maritima* FliN behaved like a single species in sedimentation equilibrium experiments, with a molecular mass (30.2 ± 2 kDa) indicating a dimer (calculated dimer molecular mass, 30.2 kDa) (Fig. 6). A dimer-tetramer equilibrium model was also tested, but the fits were not significantly better and only trace amounts of tetramer were predicted (data not shown). The *E. coli* protein also behaved like a single species in sedimentation equilibrium experiments, with a molecular mass (59.6 ± 6 kDa) indicating a tetramer (calculated molecular mass, 59.4 kDa) (Fig. 6). In velocity-sedimentation experiments, *E. coli* FliN behaved like a single species, with a sedimentation coefficient of 3.1 S, which is smaller than expected for a globular tetramer but too large for a dimer (Fig. 7). Assuming a typical level of hydration (0.5 g of water per g of protein), the shape factor for the FliN tetramer is ~1.4, indicating an elongated shape.

FliN and FliM coassemble into nascent flagella during the early step of C-ring formation (37, 42), and the proteins were shown to bind to each other in blot overlay experiments (70) and pull-down assays (67). To examine the state of purified FliM and FliN in solution, we coexpressed *T. maritima* FliN and FliM in cells and purified them by column chromatography. FliM and FliN remained together through ion-exchange, hydrophobic affinity, and gel filtration steps, which resulted in a stable, soluble complex that contained only the two proteins. The good solubility of FliM and FliN together is in contrast with the solubility of FliM alone (from either *T. maritima* or *E. coli*), which forms inclusion bodies when it is overexpressed (54, 76; unpublished results with *T. maritima* FliM). The FliM-FliN complex had an apparent molecular mass of 140 kDa as determined by gel filtration. In sedimentation equilibrium experiments the complex behaved like a single species having a molecular mass of 98.6 ± 3.5 kDa (Fig. 6). The complex appears to be quite stable, as it did not dissociate even at protein concentrations in the nanomolar range.

The experimentally estimated molecular mass (98.6 kDa) is very close to the value expected for a $FliM_1$-$FliN_4$ complex (calculated molecular mass, 98.5 kDa), but it might also fit a $FliM_2$-$FliN_2$ complex (106 kDa), given the uncertainties. To estimate the relative levels of FliM and FliN in the complex, we quantified the proteins on Coomassie blue-stained gels (Fig.