Contribution of *Salmonella typhimurium* type III secretion components to needle complex formation

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The *prgHIJK* operon encodes components of the *Salmonella typhimurium* pathogenicity island 1 type III secretion system (TTSS). Previously, *prgH* and *prgK* were shown to be required for formation of the supramolecular type III secretion needle complex (NC) [Kubori, T., *et al.* (1998) *Science* 280, 602–605]. This work indicates that all *prg* operon genes are required for NC formation. PrgH multimerizes into a distinct tetrameric-shaped structure that may be an early intermediate of NC assembly and may provide the structural foundation required for PrgK oligomerization. PrgH and PrgK, in the absence of other TTSS components, oligomerize into ring-shaped structures identical in appearance and size to the base of the NC, indicating that they are likely the major inner membrane structural components required for secretion. PrgI and PrgJ cofractionate with the NC and are secreted into the culture supernatant. NC from *prgI* and *prgJ* mutants have an identical morphology to the envelope-spanning (basal body) NC components, but are missing the external needle, indicating that PrgI and PrgJ are required for full NC assembly and are likely components of the external needle. Therefore, PrgI and PrgJ are secreted through the NC basal body, composed in part of PrgH/K and InvG/H rings, to participate in assembly of the more distal components of the NC.

Bacteria have evolved specific secretion mechanisms to facilitate the assembly of multicomponent organelles on their surfaces. The type III secretion pathway is used to assemble both flagella and virulence-associated organelles. Following organelle assembly, virulence-associated type III secretion systems (TTSS) use the type III pathway to directly transfer effector proteins into the cytosol of eukaryotic cells, were they have a variety of effects on host cell physiology. Many Gram-negative pathogens use this system to cause disease in a number of animal and plant hosts (1).

Salmonella spp. are Gram-negative bacteria that infect a variety of vertebrate hosts and cause a broad spectrum of diseases, including gastroenteritis, bacteremia, and enteric (typhoid) fever (2). *Salmonella* encode two distinct virulence-associated TTSS, which are associated with different stages of the infection process (1). The TTSS encoded on *Salmonella* pathogenicity island 1 (SPI1) is required for epithelial cell invasion and enteric pathogenesis, whereas that encoded within SPI2 is involved in intracellular survival and systemic infections (1, 3).

TTSS are composed of over 20 different structural proteins with components located within every bacterial compartment (1). Recently, the supramolecular structures of the *Salmonella typhimurium* SPI1-TTSS apparatus, termed the needle complex (NC), and the *Salmonella flexneri* TTSS apparatus, termed the secreton, were visualized by electron microscopy (4, 5). *The S. typhimurium* NC is a hollow structure about 80 nm in length composed of two distinct domains: a thin, ridged needle-like structure that extends beyond the surface of the bacterium, and a membrane-bound base structure, closely resembling the flagellar basal body, that anchors the structure to the inner and outer membranes. Biochemical analysis of purified NC revealed that three abundant proteins, InvG (a member of the secretin family), PrgH, and PrgK, were part of this complex but their association within the complex was not defined (4).

PrgH (55 kDa) and PrgK (28 kDa) are encoded within an operon of four genes (*prgHIJK*) that also encodes PrgI (6 kDa) and PrgJ (11 kDa) (6). Though not all of the genes in the operon have been formally shown to be essential to type III secretion in *Salmonella*, genes similar to *prgIJK* in *Yersinia* and *Shigella* are required for functional TTSS (6, 7). *prgH* is unique to the *Salmonella* SPI1 and the *Shigella mxi/spa* TTSS. PrgH has a hydrophobic domain that is predicted to direct its insertion and retention within the inner membrane (6, 8). Conversely, PrgK and its homologues are among the most highly conserved TTSS proteins. PrgK has a signal sequence with a canonical lipoprotein acylation site. Amino acid sequencing of PrgK isolated from the NC confirmed that it is processed at this site (4). In addition, the *Shigella* homologue, MxiJ, was shown to be acylated (9). This family of proteins also shares sequence similarity with the flagellar protein FliF. This protein inserts into the inner membrane, where it multimerizes to form the MS-ring of the flagellar basal body (10). It is likely that PrgK, like FliF, multimerizes to form a part of the basal structure of the NC.

Although PrgH and PrgK have been identified as major components of the type III secretion NC, and are likely to form the basal component of that apparatus, no data about the role of PrgI and PrgJ or their homologues exists. The *prgI/prgJ* homologues in *Yersinia* spp., *yscF/yscI*, are required for type III secretion, which suggests that PrgI and PrgJ are required for secretion in *S. typhimurium* (7). The fact that they are encoded in the same transcript with PrgH and PrgK suggested that they could play a direct role in the structure and/or assembly of the NC.

In this work, the role of the PrgHIJK in the assembly and final structure of the NC was examined.

Materials and Methods

Construction of Deletion Strains. The wild-type (WT) *Salmonella* strain (CS401) used in these studies is a streptomycin-resistant derivative of ATCC 14028s (11). Nonpolar, in-frame deletions of *prgH* (TK164), *prgI* (TK25), *prgJ* (TK26), *prgK* (TK93), and *prgH-K* (TK91) were constructed by allelic exchange. DNA flanking each of the deletions was PCR amplified with Vent DNA polymerase and cloned into the allelic exchange vector, pKAS32 (12). Allelic exchange was performed in strain CS401 as

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Abbreviations: TTSS, type III secretion system; SPI, *Salmonella* pathogenicity island; NC, needle complex; WT, wild type; TEM, transmission electron microscopy.

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described (11, 12). Greater than 95% of the coding sequence of *prgI* and *prgJ* were deleted, whereas smaller deletions were made in *prgH* (70%) and *prgK* (80%) to avoid polar effects of these deletions on expression of genes upstream (*hilD*), and downstream (*orgA*) of the operon. Deletion strains were verified by PCR and Southern blot analysis (Genius system; Roche Molecular Biochemicals). The nonpolar phenotype was verified by complementation of phenotypes with plasmids expressing the corresponding WT gene from the native *prgH* promoter on a low copy-number plasmid (see Plasmid Construction below). To facilitate isolation of NC, each of the *prg*-deletion mutations were moved into the nonflagellated *S. typhimurium* strain TK328 (strep^R SJW1399 Δ*tar flhD fljB enx*$^-$*vh2*) (13) by P22 transduction of the Amp^R-linked deletion intermediate followed by resolution of the intermediate (12) to create: TK334 (Δ*prgH*), TK335 (Δ*prgI*), TK336 (Δ*prgJ*), TK337 (Δ*prgK*), and TK338 (Δ*prgH-K*).

Plasmid Construction. The *prg* operon was amplified by PCR from pWKSSH4.0 (gift of C. Lee, Harvard Medical School, Boston) and cloned into pTK52 [pWSK29 (14) with a deletion of sequences containing the *lac* promoter and polylinker] to create pTK34 (pW*prgHIJK*). The individual *prg*-complementing plasmids: pTK30 (pW*prgH*), pTK31 (pW*prgI*), pTK32 (pW*prgJ*), and pTK33 (pW*prgK*) were derived from pTK34 by deleting the DNA flanking each gene by restriction digestion. In each case, the *prgH* promoter was left intact. To generate the arabinose-controlled *prg* expression vectors pTK43 (*prgH*), pTK48 (*prgJ*), and pTK44 (*prgK*), *prgH*, *prgJ*, and *prgK* genes were amplified by PCR and cloned into pBAD24 (15). The arabinose-controlled expression vector pTK16 (*prgH*) was generated by subcloning *prgH* into pBAD18-Kan (15). pTK42 (*prgHIJK*) was generated by subcloning the *prg* operon, minus the 5′ coding region of *prgH*, into pTK16 to recreate an intact operon under the control of the pBAD promoter. pTK53 (*prgH/K*) was generated by deleting *prgI* and *prgJ* DNA by restriction digestion from pTK42.

Analysis of Cell Lysates and Secreted Proteins. Secreted proteins were purified from either overnight cultures, as described (6), or late log phase cultures as follows: overnight cultures were diluted 1:100 into 2 ml of LB and allowed to grow for 4 h with mild aeration. Cells were removed by centrifugation at 15,000 × *g* for 5 min. The supernatant was filtered through a 0.2-μm syringe filter. Prechilled TCA was added to a final concentration of 10%, placed on ice for 30 min, then centrifuged at 15,000 × *g* for 30 min. The pellets were washed with acetone, allowed to dry, then dissolved in SDS sample buffer. For cell-associated protein fractions, bacterial cells were resuspended in an equivalent volume of sample buffer. All protein samples were boiled for 10 min before analysis by SDS/PAGE.

Arabinose-Induced Expression of PrgJ. Overnight cultures of WT (CS401) and *prg* deletion strains: ΔH (TK164), ΔI (TK25), ΔJ (TK26), and ΔK (TK93), harboring plasmid pTK48 (*prgJ* expressed from the arabinose inducible promoter) were diluted 1:10 into fresh LB. At an OD_{600} of 0.5, arabinose was added to 0.2% (vol/vol), and the cultures were incubated with shaking for an additional 2 h. Total cell lysates and supernatant fractions were prepared as described above.

Protein Analysis. SDS/PAGE and Western blot techniques were performed as described (6). Six times His-fusions were made to the N terminus of each of the Prg proteins (H, I, J, K) to facilitate their purification for antibody production. His-Prg proteins were isolated by Ni-affinity chromatography. Proteins were further purified by SDS/PAGE and then injected into rabbits at Pocono Rabbit Farm and Laboratories (Canadensis, PA). Crossreactive antibodies were depleted from the antisera by incubating with a lysate from Δ*prgH-K* (TK91) before Western blot analysis.

Preparation of NC. NC were prepared as described (4, 16) with the following modifications. Bacteria were grown with mild aeration in LB to mid-log phase (OD_{600} 0.8–1.0), pelleted, and resuspended in Solution I (0.5 M sucrose and 0.15 M Tris·HCl, pH 8.0). Lysozyme and EDTA were added to a final concentration of 0.5 mg/ml and 5 mM, respectively, and the cells were incubated with rocking at 4°C for 1 h. Triton X-100 was added to a final concentration of 1%, and the samples were incubated at 4°C for 2 h. $MgSO_4$ was then added to a final concentration of 5 mM, and lysates were rocked at 23°C for 30 min. Lysates were centrifuged at 10,000 × *g* for 20 min to remove unlysed cells and debris. Supernatants were adjusted to pH 10.5 with NaOH and then centrifuged at 80,000 × *g* in a SW28 rotor for 1 h. The pellet (preCsCl) was resuspended in TET buffer [10 mM Tris·HCl (pH 8.0), 5 mM EDTA, and 0.1% Triton X-100] and analyzed by SDS/PAGE and Western blot, or loaded onto a 30% (wt/vol) CsCl density gradient for further purification. Gradients were centrifuged at 55,000 × *g* in a SW41 rotor at RT for 16–20 h. Gradient fractions were diluted 1:10 with TET buffer and pelleted at 80,000 × *g* at 4°C for 1 h. Pellets were washed once with TET and resuspended in 20 μl TET for transmission electron microscopy (TEM) analysis or in SDS sample buffer for SDS/PAGE and Western blot analysis.

Isolation of Prg Protein Complexes from *E. coli*. Recombinant *E. coli* (DH5α) expressing *prg* genes: *prgH* (pTK16), *prgK* (pTK44), *prgH/K* (pTK53), and *prgHIJK* (pTK42), were diluted 1:100 from overnight cultures into fresh LB and grown to an OD_{600} of 0.5. Arabinose was added to a final concentration of 0.2% (vol/vol), and the cultures were grown an additional 2 h. Prg complexes were isolated from *E. coli* by using a protocol similar to the NC isolation protocol described above with the following modifications: following the detergent lysis step as described above, $MgSO_4$ (10 mM final), DNase (5 μg/ml final), and RNase (10 μg/ml final) were added, and the lysate was incubated at 30°C for 30 min. Unlysed cells were removed by centrifugation, and the pH of the lysate was adjusted to 10.5 with NaOH. The lysate was then filtered through a 0.45-μm syringe filter to remove any remaining particulate matter. Protein complexes were pelleted as above by high-speed centrifugation. The resulting protein pellets were washed twice by resuspention in TET solution followed by high-speed centrifugation as before. The final pellet was resuspended in a small volume of TET (10–30 μl). Samples were pure enough at this stage for direct visualization by TEM. The CsCl purification step was omitted.

Electron Microscopy. NC preparations and isolated Prg complexes were negatively stained with 1% phosphotungstic acid (pH 7.0) and applied directly to 0.5% formvar-coated 300 mesh copper grids. Samples were observed with a JEM-1200EXII transmission electron microscope (JEOL). Micrographs were taken at an accelerating voltage of 80 kV.

Results

PrgHIJK Are All Required for Type III Secretion and Invasion of Epithelial Cells. When grown in culture, *S. typhimurium* secretes a number of proteins into the culture supernatant (17, 18). To determine the role of the individual proteins encoded by the *prgHIJK* operon in type III secretion, in-frame deletions of each individual gene within the operon were created by allelic exchange. Analysis of culture supernatants from each individual *prg* deletion strains revealed that all of the genes are essential for SPI1 type III secretion (Fig. 1). Consistent with the secretion results, each of the deletion strains had a 50- to 100-fold defect in the ability to invade HeLa cells as measured by gentamicin

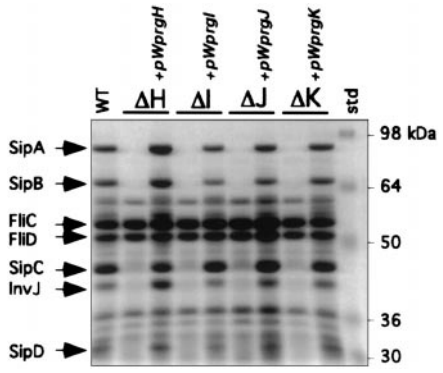

Fig. 1. Each of the *prgs* is required for type III secretion. SDS/PAGE analysis of secreted proteins from WT *S. typhimurium* (CS401), isogenic strains harboring nonpolar deletions of each of the *prg* genes; ΔH (TK164), ΔI (TK25), ΔJ (TK26), ΔK (TK93) and each of the corresponding complemented strains; ΔH + pWprgH (pTK30), ΔI + pWprgI (pTK31), ΔJ + pWprgJ (pTK32), and ΔK + pWprgK (pTK33). The samples were prepared from overnight cultures grown with mild aeration. The identity of proteins secreted by the SPI1 TTSS and flagellar system are indicated on the left.

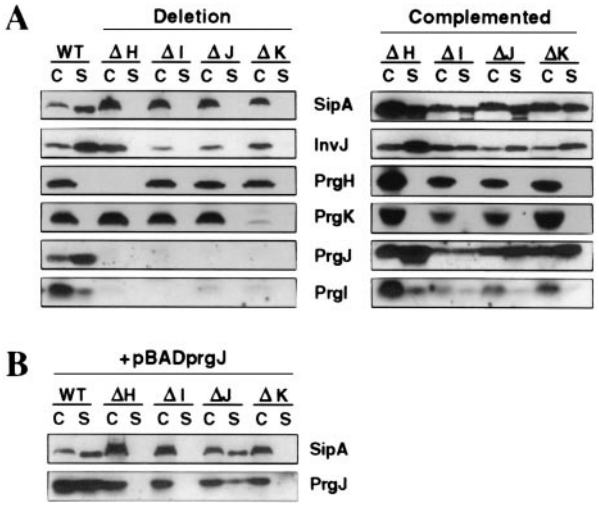

Fig. 2. PrgI and PrgJ are found in the culture supernatant. (*A*) Western blot analysis of whole cell lysates (C) and culture supernatants (S) from WT *S. typhimurium* (CS401), the isogenic *prg* deletion strains: ΔH (TK164), ΔI (TK25), ΔJ (TK26), ΔK (TK93), and each of the corresponding complemented strains: ΔH + pWprgH (pTK30), ΔI + pWprgI (pTK31), ΔJ + pWprgJ (pTK32), ΔK + pWprgK (pTK33). Cells were grown to late log phase with mild aeration. The amounts of whole cell lysate (C) and supernatant (S) fractions loaded per lane represent equivalent amounts of total culture volume. Blots were probed with polyclonal antibodies directed against SipA, InvJ, PrgH, PrgI, PrgJ, and PrgK as indicated. (*B*) Western blot analysis of whole cell lysates, and culture supernatants from WT (CS401) and *prg*-deletion strains: ΔH (TK164), ΔI (TK25), ΔJ (TK26), ΔK (TK93) harboring plasmid pBADprgJ (pTK32). Equivalent amounts of whole cell lysate and supernatant fractions were loaded per lane. Blots were probed with anti-PrgJ and anti-SipA polyclonal antiserum as indicated.

protection assay (data not shown). Deletions were confirmed to be nonpolar through complementation of defects by single genes (corresponding to each deletion), carried on a low copy plasmid (pWSK29) under the control of the native *prgH* promoter (Fig. 1, and data not shown). These data show that *prgHIJK* are all required for type III secretion and for invasion of epithelial cells *in vitro*.

PrgI and PrgJ Are Found in the Culture Supernatant. To determine the subcellular localization of PrgI and PrgJ, cell-associated and supernatant fractions isolated from late logarithmic phase cultures of WT and *prg*-deletion strains were analyzed by SDS/PAGE and Western blot (Fig. 2*A*). PrgI and PrgJ were detected in both the cell-associated and secreted fractions from WT cells. Similar results were found for the secreted proteins SipA and InvJ (19). In each of the *prg*-deletion strains, PrgI and PrgJ were not detectable in the supernatant fractions, whereas in the cell-associated fractions, PrgJ was undetectable and PrgI levels were greatly reduced. The secreted proteins InvJ and SipA were detected only in the cell-associated fractions of each of the *prg*-deletion mutants (Fig. 2*A*). Complementation of each of the *prg*-deletion strains resulted in detectable levels of PrgI and PrgJ in both the cell-associated and secreted fractions (Fig. 2*A*-complemented). This suggests that either the synthesis or stability of these two proteins depends on the other three *prgs*. Because PrgH and PrgK levels are not significantly altered, and PrgI and PrgJ levels are restored by single gene complementation in each of the deletion strains, the absence of PrgI and PrgJ is not the result of polar effects of the *prg*-deletions. The effects of these deletions on the level of PrgI and PrgJ may be because of a decrease in the level of translation or posttranslational stability of these two proteins.

The *prgH* deletion strain complemented with a multicopy plasmid showed an increase over WT in the levels of Prg, Inv, and Sip proteins in both cell-associated and secreted fractions (Fig. 2*A*-complemented). In addition, the secretion profile of this strain also showed increased secretion of all type III secreted proteins (Fig. 1*A*). This effect is similar to what has been previously observed for complementation of a *prgH*::Tn*phoA* insertion mutant with a multicopy plasmid (6).

***prgH*- and *prgK*-Dependent Secretion of PrgJ.** The lack of secretion of PrgI and PrgJ in each of the *prg*-deletion strains may simply reflect a lack of synthesis and/or stability of these proteins in the absence of a functional type III secretion apparatus. To test whether *prgH*, *prgI*, and *prgK* were required for PrgJ secretion in the absence of synthesis or stability issues, *prgJ* was expressed from the arabinose promoter in WT and *prg*-deletion strains. Upon overexpression, PrgJ was detected in both the cell-associated and supernatant fractions from WT and Δ*prgJ* (Fig. 2*B*). However, in Δ*prgH*, Δ*prgI*, and Δ*prgK*, PrgJ was detected only in the cell-associated fraction. The secretion profile of SipA was identical to that of PrgJ. This finding strongly suggests that secretion, in addition to the synthesis and/or stability of PrgJ, requires PrgH, PrgI, and PrgK. Similar overexpression experiments were attempted with PrgI; however, whereas regulated *prgI* expression from the pBAD plasmid complemented the secretion defect of the *prgI* deletion strain, we were unable to detect PrgI accumulation in *prg* deletion strains despite its overproduction (data not shown). This may be because of intrinsic instability of PrgI in the absence of other *prg* operon components.

***prgI* and *prgJ* Are Required for the Formation of an Intact NC Structure.** PrgH and PrgK were found to copurify with the NC (4). Because *prgI* and *prgJ* are coordinately expressed with *prgH* and *prgK* and are required for SPI1-dependent secretion, we hypothesized that they may play a direct role in the assembly, or may be structural components of the NC. NC were isolated from WT *S. typhimurium* (SJW1399) according to the protocol by Kubori *et al.* (4) with modifications as noted. Western-blot analysis of the postCsCl NC preparation was used to show that PrgI and PrgJ, but not InvJ, cofractionate with PrgH and PrgK (Fig. 3*A*). TEM analysis of the preparation confirmed that it was highly enriched for NC (Fig. 3*C*). These observations are consistent with the hypothesis that PrgI and PrgJ are components of the NC.

prgI and *prgJ* mutants were analyzed for the formation of NC

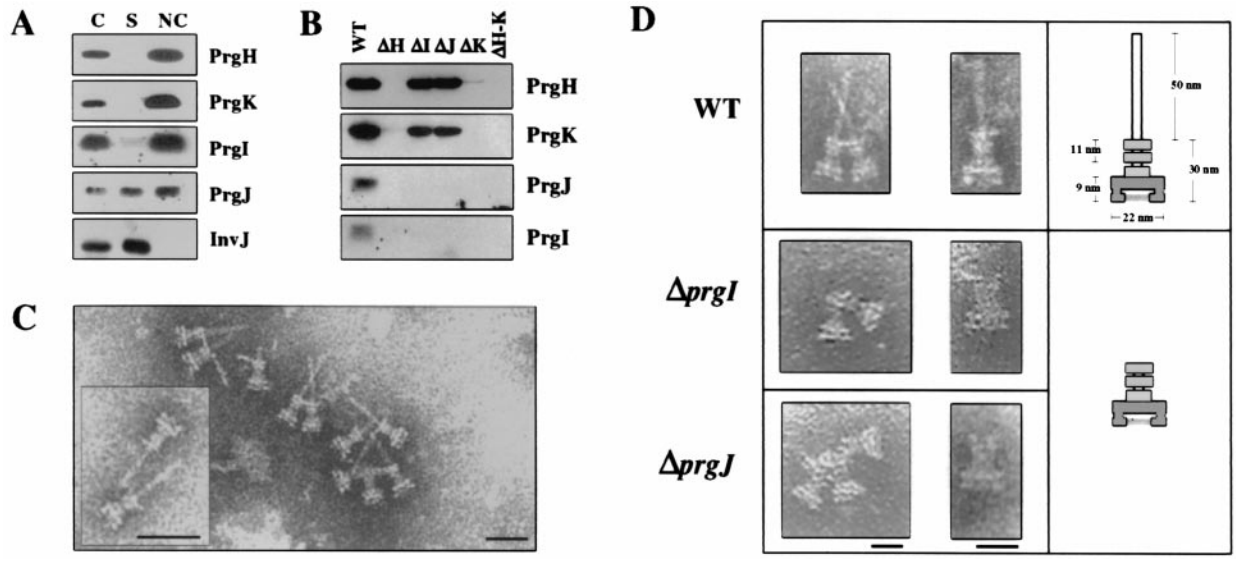

Fig. 3. *prgI* and *prgJ* are required for needle structure formation. (*A*) Western-blot analysis of whole cell lysate (C), culture supernatant (S), and postCsCl needle complex fractions (NC) isolated from WT *Salmonella* (TK328). The amount of C and S fractions loaded represents equivalent amounts of total culture volume (0.2 ml), the NC represents 25 ml of culture. The blot was probed with polyclonal antiserum against PrgH, PrgI, PrgJ, PrgK, and InvJ as indicated. (*B*) Western blot analysis of PreCsCl NC fractions isolated from WT and *prg* deletion strains: WT (TK328), ΔH (TK334), ΔI (TK335), ΔJ (TK336), ΔK (TK337), ΔH-K (TK338). The blot was probed with polyclonal antiserum against PrgH, PrgI, PrgJ, and PrgK as indicated. (*C*) Electron micrographs of NC isolated from WT (TK328). Complexes came from same fraction (NC) analyzed in *A*. Samples were negatively stained with 2% PTA (pH 7.2) and observed under a JEM-1200EXII transmission electron microscope. Approximate sizes of the NC are listed in the schematic (*D*) ($n = 30$). (Scale bar, 50 nm.) (*D*) Electron micrographs of partial NC isolated from WT (TK328), ΔprgI (TK335), and ΔprgJ (TK336) deletion mutants. A schematic comparing the size and morphology of these structures to WT structures is also shown. Approximate sizes are given in schematic ($n = 30$). (Scale bars, 50 nm and 25 nm.)

structures containing PrgH and PrgK. PreCsCl NC fractions isolated from *prgI* and *prgJ* deletion mutants contained similar amounts of PrgH and PrgK as fractions from WT highly enriched for NC (Fig. 3*B*, ΔI and ΔJ). In contrast, Western blot analysis of preCsCl NC preparations from both *prgH* and *prgK* deletion mutants indicated greatly reduced levels of both PrgH and PrgK (Fig. 3*C*, ΔH and ΔK). This finding indicates that PrgH and PrgK are both required for the formation of a stable multimeric structure of similar density to the NC.

Examination of CsCl purified NC fractions from the *prgI* and *prgJ* deletion strains by TEM revealed partial NC containing most of the putative membrane-bound basal body-like components (inner rings, rod, and outer rings), but missing the needle (Fig. 3*D*). The majority of these structures were found in clusters, associated through interactions of the outer membrane rings. The sizes of these structures are identical to that of the intact NC missing the needle (Fig. 3*D*). It should be noted that the size estimates for the NC are smaller than those previously reported (4). These results indicate that *prgI* and *prgJ* are not required for the assembly of the membrane-bound components (basal body) of the NC. Additionally, they suggest that PrgI and PrgJ either may be structural components of the needle itself, or may be required for its assembly.

PrgH Forms a Stable Tetrameric-Shaped Multimer. In addition to the partial NC structures observed in preparations from Δ*prgI* and Δ*prgJ*, tetrameric-shaped structures, which may represent an intermediate of NC assembly, were also observed. These structures were also seen in NC preparations from WT *Salmonella* (Fig. 4*D*) as well as from Δ*prgI*, Δ*prgJ*, and Δ*prgK* mutants (data not shown). Because PrgH and PrgK were present at WT levels in NC preparations from *prgI* and *prgJ* deletion mutants, this suggested that one of these proteins may oligomerize to form this tetrameric structure. To determine whether PrgH or PrgK can form an oligomeric structure independent of other TTSS components, each was independently expressed in *E. coli* (DH5α). PrgH- and PrgK-containing complexes were isolated from *E. coli* by using a modified NC preparation protocol (i.e., detergent lysis followed by sedimentation). Suprisingly, NC isolation resulted in highly purified fractions containing either PrgH or PrgK (Fig. 4*A*). Sedimentation of PrgH and PrgK by ultracentrifugation following detergent extraction indicates that these proteins must exist as oligomeric complexes or aggregates. Purified oligomeric PrgH and PrgK represented only a small percentage (approx. 1–5%) of the total expressed in *E. coli* (Fig. 4*A*), and coexpression of PrgH and PrgK (+*prgH*/*prgK*) did not increase the percentage of oligomeric PrgH or PrgK isolated (Fig. 4*A*)

PrgH complexes purified from *E. coli* were further analyzed by Western blot. Approximately half of the PrgH-containing fraction formed an SDS-resistant complex detected as a higher migrating band in SDS/PAGE (Fig. 4*B*). When the sample was boiled, PrgH was completely dissociated to a size consistent with its known monomeric molecular mass (52 kDa). Similar analysis of purified fractions from *E. coli* expressing *prgK* or *prgH*/*K* indicate that PrgK is not present in SDS-resistant complexes. In addition, the presence of PrgK does not alter the amount of SDS-resistant PrgH (data not shown). Therefore, PrgH forms these complexes independently of PrgK.

NC preparations from *E. coli* expressing *prgH* or *prgK* were examined by TEM to establish the morphology of these oligomeric structures. Visualization of negatively stained preparations of PrgK revealed bar-like structures approximately 3–5 nm wide, with lengths varying between 10 and 50 nm (data not shown). Although it can be concluded that PrgK forms oligomeric complexes when over-expressed in *E. coli*, the lack of a consistently sized structure greatly limits any conclusions that can be made regarding its physiological relevance. In contrast, visualization of negatively stained preparations of PrgH revealed defined structures that appeared to be tetrameric with a square dimension of approximately 10 nm (Fig. 4*C*). These structures were identical to structures seen in preparations of purified NC from WT *S. typhimurium*, Δ*prgI*, Δ*prgJ*, and Δ*prgK* (Fig. 4*D*, and

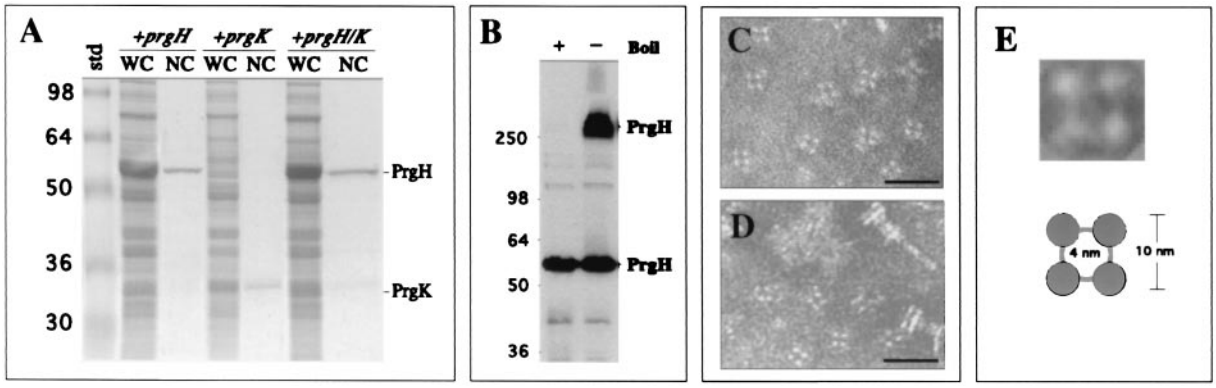

Fig. 4. Prg complex isolation from *E. coli*. (*A*) SDS/PAGE of whole cell lysates (WC) and needle complex fractions (NC) prepared from recombinant *E. coli* (DH5α) expressing *prgH* (pTK43), *prgK* (pTK44), or *prgH*/*prgK* (pTK53). (*B*) Western blot analysis of PrgH isolated from *E. coli* (from *A*). The sample was resuspended in SDS loading buffer and boiled (+) or directly loaded (−) onto the gel. Blot was probed with polyclonal antiserum to PrgH. (*C* and *D*) Electron micrographs of (*C*) PrgH isolated from *E. coli*, and (*D*) NC isolated from WT *S. typhimurium*. In both *C* and *D*, numerous tetrameric-shaped structures can be seen. (Scale bar, 50 nm.) (*E*) Enlarged image of one of the tetramers from *C* with a schematic showing an approximate size of 10 nm × 10 nm (n = 25). (Scale bar, 50 nm.)

data not shown). These data indicate that PrgH forms a multimeric structure independent of other type III secretion components and may represent an early intermediate in NC assembly.

PrgH and PrgK Form the Inner Membrane Rings of the NC. Although PrgH alone can oligomerize into a definable structure, it is likely that PrgH interacts with PrgK as both proteins are required for the formation of the basal body component of the NC (Fig. 3*B*). These data suggest that PrgH and PrgK may interact to form an intermediate of NC assembly. To test this hypothesis, *E. coli* expressing both *prgH* and *prgK* were analyzed to determine whether PrgH and PrgK together formed a definable structure. Again, NC preparations from *E. coli* expressing *prgH* and *prgK* yielded highly purified fractions containing predominately these two proteins (Fig. 4*A*, +*prgH*/*K*). Visualization of these samples by TEM revealed three distinct structures: tetrameric-shaped structures identical to the PrgH multimer (compare Fig. 5*A* and 4*C*), ring-shaped and rectangular structures (Fig. 5 *A* and *B*). The ring structures had outer and inner diameters of approximately 21 nm and 12 nm, respectively. The rectangular-shaped structures were approximately 21 nm in width and 9 nm in height. These two structures most likely represent top and side views of the same structure. Some oblique views were also present (Fig. 5*B*). The rectangular structures (side view) were most often visualized in pairs. This is probably because of strong hydrophobic interactions between the structures, often seen with purified membrane proteins (20). The rectangular structures (side view) could be grouped into two classes: structures which appeared as two parallel bars, and structures which had a complete bar and a bar with a central gap (staple). These structures are virtually identical in size and shape to the base rings of the NC (Fig. 5*C*). In addition, the diversity in shape (parallel bars vs. staple) of these structures mimics the differences seen in the base structures of purified NC (Fig. 5*C*). Identical structures were obtained from *E. coli* expressing *prgHIJK*. These data indicate that PrgH and PrgK form the base rings of the NC and that their ability to do so is independent of all other type III secretion system components, including PrgI and PrgJ.

Discussion

The recent visualization by Aizawa and colleagues (4) of the SPI1 type III secretion apparatus from *S. typhimurium* indicated that it had a defined structure, morphologically analogous to the flagellar basal body, termed the needle complex (NC). These authors also determined that *invG*, *prgH*, and *prgK* were required for production of the NC. The abundance of InvG, PrgH, and PrgK in subcellular fractions enriched for NC suggested that they were major structural components (4). InvG, a member of the PulD family of pore forming outer membrane proteins (secre-

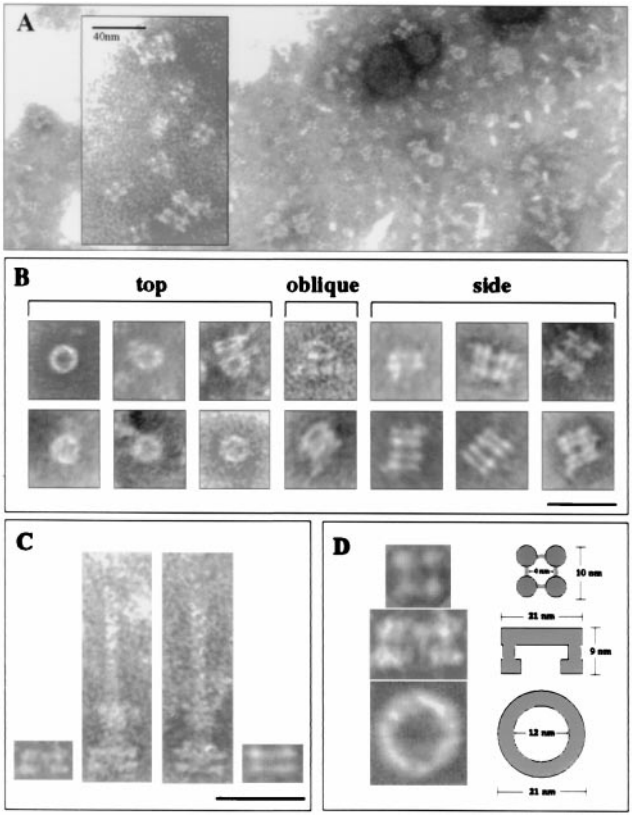

Fig. 5. PrgH and PrgK form the inner membrane rings of the NC. (*A* and *B*) Representative electron micrograph images of PrgH/PrgK complexes from recombinant *E. coli* (DH5α) expressing *prgH*/*prgK* (pTK42) or *prgHIJK* (pTK53). (*B*) Electron micrographs of individual ring structures (top), rectangular structures (side), and oblique view of same structures. (Scale bar, 50 nm.) (*C*) Comparison of two different side views of the PrgH/K complex with NC isolated from WT *Salmonella*. Note that the differences seen in these structures are reflected in the differences seen in the base structure of the NC. Scale bar, 50 nm. (*D*) Schematic comparing the approximate size and shape of the isolated Prg components: tetramer, 10 nm (n = 25); rectangular (side), width 21 nm, height 9 nm (n = 27); ring (top), outer diameter 21 nm, inner diameter 12 nm (n = 30).

tins), forms an oligomeric ring structure in *E. coli* when expressed with InvH (21). These rings have an estimated diameter and pore size consistent with their classification as the outer membrane rings of the NC. Based on their membrane localization and their similarity to flagellar proteins, PrgH and PrgK were predicted to form the cytoplasmic base of the NC.

This work provides more detail about the protein components and assembly of the NC. Other proteins encoded in the *prg* operon, PrgI and PrgJ, were shown to be involved in NC formation. These proteins were found in subcellular fractions enriched for NC. Unlike PrgH, PrgK, and InvG, which are all membrane-associated proteins, PrgI and PrgJ are secreted into the culture supernatant. Secretion of PrgI and PrgJ may be analogous to the secretion into the culture supernatant of more distal components of the flagellar hook and basal body during flagellar assembly (18). Secretion of PrgJ, and most likely PrgI, requires *prgH* and *prgK*, suggesting that assembly of the multiprotein NC occur through ordered secretion, polymerization, and assembly, analogous to flagellar morphogenesis. Levels of PrgI and PrgJ were markedly reduced in the absence of one another, indicating that they may physically interact. Because a structure corresponding to the membrane-bound components of the NC missing the needle were isolated from *prgI* and *prgJ* mutants, this suggested that they may be components of the needle itself. Alternatively, PrgI and PrgJ may be essential linkers between the InvG/InvH outer membrane ring and another polymeric protein(s) that forms the needle.

E. coli expression studies of PrgH indicate that it can multimerize into a defined tetrameric-like structure. This structure is also visualized in the NC fraction from WT *S. typhimurium*, Δ*prgI*, Δ*prgJ*, and Δ*prgK*, suggesting that it may be an intermediate in NC assembly. The PrgH tetramer-like structure may form a base in the cytoplasmic membrane for assembly of other NC components. In support of this hypothesis, PrgK does not form a definitive structure when expressed alone in *E. coli* and the ability to detect PrgK in NC preparations from *S. typhimurium* requires *prgH*, suggesting that PrgH may be directly involved in the oligomerization of PrgK.

In NC preparations from *Salmonella,* the presence of PrgK greatly increases the amount of multimeric PrgH. However, coexpression of *prgH* and *prgK* in *E. coli* does not significantly increase the amount of PrgH-containing complexes, suggesting that PrgK alone does not effect the level of PrgH multimerization. Therefore, it is possible that the decreased level of multimeric PrgH isolated from Δ*prgK* represents a stable pool of PrgH tetrameric complex independent of other membrane-associated type III secretion components such as PrgK and InvG. Under WT conditions in the presence of a core set of membrane-bound type III secretion components, PrgH may be incorporated into a larger, more stable, and more abundant complex, thereby increasing the amount of multimeric PrgH that can be isolated.

Expression of both *prgH* and *prgK* in *E. coli* results in the formation of a ring structure morphologically identical to the base of the NC, indicating that PrgH and PrgK can form this structure independent of other TTSS apparatus components. The PrgH/K complex had a width of approximately 22 nm and a height of 9 nm. These dimensions are identical to measurements made of the base rings of the NC. In addition, the PrgH/K complex ring structure has an inner diameter of approximately 12 nm, which is consistent with the idea that the NC contains a central secretion pore. Interestingly, FliF, the flagellar homologue of PrgK, polymerizes to form the cytoplasmic (MS) rings of the flagellar basal body (10). The central pore contained within FliF houses cytoplasmic components, conserved between the flagellar and virulence associated type III secretion machinery. These components are required for secretion of more distal apparatus components across the inner membrane (22). It is possible that the PrgH/PrgK complex serves a similar role and houses the homologous cytoplasmic secretion components of the SPI-1 type III secretion apparatus.

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1. Hueck, C. J. (1998) *Microbiol. Mol. Biol. Rev.* **62,** 379–433.
2. Mandell, G. L., Douglas, R. G. & Bennett, J. E. (1995) *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases* (Churchill Livingstone, New York), 4th Ed., p. 2.
3. Hensel, M., Shea, J. E., Gleeson, C., Jones, M. D., Dalton, E. & Holden, D. W. (1995) *Science* **269,** 400–403.
4. Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galan, J. E. & Aizawa, S. I. (1998) *Science* **280,** 602–605.
5. Blocker, A., Gounon, P., Larquet, E., Niebuhr, K., Cabiaux, V., Parsot, C. & Sansonetti, P. (1999) *J. Cell Biol.* **147,** 683–693.
6. Pegues, D. A., Hantman, M. J., Behlau, I. & Miller, S. I. (1995) *Mol. Microbiol.* **17,** 169–181.
7. Allaoui, A., Schulte, R. & Cornelis, G. R. (1995) *Mol. Microbiol.* **18,** 343–355.
8. Leclerc, G. J., Tartera, C. & Metcalf, E. S. (1998) *Infect. Immun.* **66,** 682–691.
9. Allaoui, A., Sansonetti, P. J. & Parsot, C. (1992) *J. Bacteriol.* **174,** 7661–7669.
10. Ueno, T., Oosawa, K. & Aizawa, S. (1992) *J. Mol. Biol.* **227,** 672–677.
11. Rakeman, J. L., Bonifield, H. R. & Miller, S. I. (1999) *J. Bacteriol.* **181,** 3096–3104.
12. Skorupski, K. & Taylor, R. K. (1996) *Gene* **169,** 47–52.
13. Gillen, K. L. & Hughes, K. T. (1991) *J. Bacteriol.* **173,** 2301–2310.
14. Wang, R. F. & Kushner, S. R. (1991) *Gene* **100,** 195–199.
15. Guzman, L. M., Belin, D., Carson, M. J. & Beckwith, J. (1995) *J. Bacteriol.* **177,** 4121–4130.
16. Aizawa, S. I., Dean, G. E., Jones, C. J., Macnab, R. M. & Yamaguchi, S. (1985) *J. Bacteriol.* **161,** 836–849.
17. Hueck, C. J., Hantman, M. J., Bajaj, V., Johnston, C., Lee, C. A. & Miller, S. I. (1995) *Mol. Microbiol.* **18,** 479–490.
18. Komoriya, K., Shibano, N., Higano, T., Azuma, N., Yamaguchi, S. & Aizawa, S. I. (1999) *Mol. Microbiol.* **34,** 767–779.
19. Collazo, C. M., Zierler, M. K. & Galan, J. E. (1995) *Mol. Microbiol.* **15,** 25–38.
20. Linderoth, N. A., Simon, M. N. & Russel, M. (1997) *Science* **278,** 1635–1638.
21. Crago, A. M. & Koronakis, V. (1998) *Mol. Microbiol.* **30,** 47–56.
22. Fan, F., Ohnishi, K., Francis, N. R. & Macnab, R. M. (1997) *Mol. Microbiol.* **26,** 1035–1046.