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A Subassembly of R27-Encoded Transfer Proteins Is Dependent on TrhC Nucleoside Triphosphate-Binding Motifs for Function but Not Formation

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The transfer of plasmid DNA molecules between bacterial cells is achieved by a large array of conjugative transfer proteins which assemble into both cytoplasmic and membrane-associated complexes. TrhC is a membrane-associated protein that is required for the transfer of the IncHI1 resistance plasmid R27. Homologous proteins are encoded in all known conjugative systems, and each contains characteristic nucleoside triphosphate (NTP)-binding domains. An assembly of R27-encoded proteins was previously visualized by use of a TrhC-green fluorescent protein fusion, which appeared as discrete membrane-associated fluorescent foci. We have utilized this experimental system to determine the requirements for assembly of this TrhC-associated protein complex, and we found that 12 of the other 18 R27 transfer proteins are required for focus formation. An individual focus possibly represents a subassembly comprised of some or all of these transfer proteins. These data support the notion that the transfer apparatus is a multicomponent structure. In contrast, substitutions and deletions within TrhC NTP-binding motifs had minor effects on focus formation, but these mutations did affect plasmid transfer and bacteriophage susceptibility. These results indicate that TrhC requires intact NTP-binding motifs to function during conjugative transfer but that these motifs are not essential for the assembly of TrhC into a complex with other transfer proteins.

Conjugative transfer is classified as type IV secretion (1) and is one of the principal mechanisms for horizontal gene transfer between bacteria. The proteins which facilitate the conjugative transfer of plasmid DNA represent a dynamic module that is functional in the cytosol, cell envelope, and extracellular environment. Besides the pilus, no distinguishable superstructures have been visualized in vivo for the conjugative apparatus, although several macromolecular complexes have been proposed to exist. These include the cytoplasmic relaxosome, responsible for DNA processing prior to and during transfer (18, 23); the membrane-associated mating pair formation complex (Mpf), responsible for construction of the pilus and for DNA transfer (12); and a multimeric coupling protein which links the relaxosome to the Mpf complex (6, 10, 27). Despite the absence of a readily visible transmembrane apparatus during electron microscopy such as that visualized for type III secretion systems (3), several studies have identified specific interactions between type IV secretion proteins or have identified functional subgroups indicative of protein assemblies (6, 12, 13, 21, 33).

The individual proteins that comprise the different conjugative systems have conserved motifs, and in particular, Walker-type nucleoside triphosphate (NTP)-binding domains (motif A, GxxGxGKS/T; motif B, hhhhDE, where h indicates a hydrophobic residue [26]) are the most common. Each member of the TraG family of coupling proteins has these domains, but none has been shown to hydrolyze ATP in vitro (27). ATP hydrolysis is predicted to be an in vivo activity of the coupling proteins, resulting in structural rearrangements within the inner membrane gate and possibly energizing substrate translocation through the inner membrane to the Mpf complex (8, 9). NTP-binding motifs also partly define members of the VirB4 family of inner membrane-associated transfer proteins, and it has been speculated that these motifs hydrolyze ATP to power either the assembly of the transfer apparatus or the translocation of substrate (35). Similar to the in vivo results with purified coupling proteins, preparations of the VirB4-related proteins TrbE and TrwK, encoded by plasmids RP4 and R388, respectively, also did not hydrolyze ATP or GTP (24). ATP hydrolysis by TrbE and VirB4 may occur under in vivo conditions, since the NTP-binding motifs are essential for the functioning of both proteins (2, 5, 24). The precise role of the NTP-binding motifs of VirB4-related proteins in the conjugative donor apparatus is ill-defined.

Our study focused on the VirB4-related protein TrhC, an inner membrane-associated Mpf protein required for the conjugative transfer of the large IncHI1 resistance plasmid R27, originally identified in *Salmonella enterica* serovar Typhi (20, 28). Previously, we created a TrhC-green fluorescent protein (GFP) fusion construct and were able to visualize the cellular location and observe the temperature-dependent assembly of the TrhC-containing protein complexes (7). TrhC was present in membrane-associated protein complexes, visualized as distinct fluorescent foci at the cell periphery. For the present study, our aims were to identify the R27-encoded proteins that are required for the formation of TrhC-containing protein complexes and to characterize the role of the Walker NTP-binding motifs within TrhC in protein complex assembly, DNA transfer, and phage susceptibility.

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TABLE 1. Plasmids and *E. coli* strains used for this study

Strain or plasmid	Description	Selective marker(s)[a]	Source or reference
E. coli strains			
DY330	W3110 Δ*lacU169 gal490* λ*c1857* Δ(*cro-bioA*); spontaneous Nal^r or Rif^r	Nal or Rif	34
RG192	*ara leu lac*	Rif	30
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* lac [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*]	Tc	Stratagene
Plasmids			
pDT1942	Derepressed transfer mutant of R27	Tc, Km	22
pDT2953	pDT1942 with mini::Tn*10* inserted into *trhU*	Tc, Km, Cm	25
pDT2957	pDT1942 with mini::Tn*10* inserted into *trhC*	Tc, Km, Cm	32
pDT2959	pDT1942 with mini::Tn*10* inserted into *trhW*	Tc, Km, Cm	25
pDT2969	pDT1942 with mini::Tn*10* inserted into *trhC*	Tc, Km, Cm	32
pDT2970	pDT1942 with mini::Tn*10* inserted into *trhE*	Tc, Km, Cm	25
pDT2971	pDT1942 with mini::Tn*10* inserted into *trhB*	Tc, Km, Cm	25
pDT2978	pDT1942 with mini::Tn*10* inserted into *trhC*	Tc, Km, Cm	32
pDT2981	pDT1942 with mini::Tn*10* inserted into *trhK*	Tc, Km, Cm	25
pDT2984	pDT1942 with mini::Tn*10* inserted into *trhH*	Tc, Km, Cm	19
pDT2987	pDT1942 with mini::Tn*10* inserted into *trhF*	Tc, Km, Cm	19
pDT2988	pDT1942 with mini::Tn*10* inserted into *trhN*	Tc, Km, Cm	25
pDT2989	pDT1942 with mini::Tn*10* inserted into *traG*	Tc, Km, Cm	19
pDT2991	pDT1942 with mini::Tn*10* inserted into *trhG*	Tc, Km, Cm	19
pDT2995	pDT1942 with mini::Tn*10* inserted into *traI*	Tc, Km, Cm	19
pJEG51	pDT1942 with *cat* inserted into *traJ*	Tc, Km, Cm	19
pJEG94	pDT1942 with *cat* inserted into *trhR*	Tc, Km, Cm	19
pJEG104	pDT1942 with *cat* inserted into *traH*	Tc, Km, Cm	19
pJEG115	pDT1942 with *cat* inserted into *trhY*	Tc, Km, Cm	19
pGUN110	pDT1942 with *cat* inserted into *trhA*	Tc, Km, Cm	20
pMS119EH	Cloning vector, P_{tac}-*lacI*$^{q-}$, pMB1 origin of replication	Ap	29
pMWG36	$TrhC_{His6}$ expression vector (pMS119EH)	Ap	7
pMWG191	TrhC-GFP expression vector (pMS119EH)	Ap	7
pMWG253	pDT1942 with *cat* inserted into *trhL*	Tc, Km, Cm	20
pMWG265	pDT1942 with *cat* inserted into *trhV*	Tc, Km, Cm	20

[a] Antibiotic resistance markers: Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Cm, chloramphenicol; Nal, nalidixic acid; Rif, rifampin.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. The *Escherichia coli* strains, expression constructs, and R27 transfer mutants used for this study are presented in Table 1. Strains were grown in Luria-Bertani medium (Lennox formulation; Difco Laboratories) at 28°C because of the temperature-sensitive nature of the R27 conjugative apparatus (7). Appropriate antibiotics were added at the following final concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; nalidixic acid, 30 μg ml^{-1}; rifampin, 20 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; and chloramphenicol, 16 μg ml^{-1}. For the visualization of fluorescence patterns in live cells, liquid cultures were grown at 28°C to an optical density at 600 nm of ~0.6, induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h while incubation continued at 28°C, harvested by centrifugation, washed in phosphate-buffered saline (Oxoid), and then imbedded in a translucent nutrient agar slab on a microscope slide, and still images were captured under UV illumination (7). The enumeration of fluorescent foci was performed as described previously (7, 11). For the determination of conjugative transfer frequencies in complementation mating experiments, donor cells (*E. coli* RG192 or DY330R plus R27 *trhC* transfer mutant plus *trhC*-encoding expression vector) were mixed with recipient cells (DY330N) for 18 h and plated on selective media as previously described (19, 31). Hgal bacteriophage spot tests were also performed as previously described (7).

Site-directed mutagenesis of Walker A- and B-encoding regions of TrhC. Plasmids pMWG191 (encoding *trhC-gfp*) and pMWG36 (encoding $trhC_{His6}$) served as templates for site-directed mutagenesis experiments. For the introduction of substitutions and deletions into *trhC*, a complementary pair of oligonucleotide primers containing mismatches or deletions in comparison to the template sequence was used in a thermocycling reaction with *PfuTurbo* (Stratagene) to synthesize full-length DNA molecules from the plasmid templates. These primers were based upon the following sequences from the *trhC* sense strand: 5′GCAACGTCTGGTGCTGGTAAATCATTCTGGGTCGCATAC (Walker A) and 5′CGGGTTGTTCATACTTGATGAGGCATGGGAGTATATCCG TCCG (Walker B), where the underlined segments represent regions containing mutation-specific mismatches or deletions (detailed below). The parental wild-type plasmid DNA was selectively destroyed by *Dpn*I (Invitrogen), which recognizes and cleaves methylated DNA substrates. The newly synthesized, unmethylated DNA containing mutations was transformed into XL1-Blue *E. coli* ultracompetent cells (Stratagene) for circularization and propagation. The plasmid constructs were sequenced in the target area (using primers Walker B, 5′ACACCTTCAGACTGTCGTTCTG, and Walker A, 5′CTCCATCAGGGTC AAATGCATC) and were analyzed by restriction digestion to ensure that mutated constructs of the proper sizes had been generated. All pMWG191 wild-type and mutation constructs were then transformed into RG192 cells containing pDT2969 for fluorescence assays, whereas pMWG36 wild-type and mutation constructs were transformed into RG192 cells containing pDT2957, pDT2969, or pDT2978 for conjugative transfer complementation and bacteriophage propagation assays. For determination of the level of TrhC or TrhC-GFP production from the mutant constructs, an immunoblot analysis of whole-cell lysates was conducted with polyclonal TrhC antiserum and was processed as previously described (7).

RESULTS

Specific R27 transfer proteins are required for assembly of the TrhC-GFP complex. Previously, when TrhC-GFP was produced from the R27 derivative pDT3048 (including an in-frame insertion of *gfp* with *trhC* to create a *trhC-gfp* fusion), fluorescent foci representing transfer protein assemblies were observed at random positions in the cell membrane (7). The pDT3048 construct was still capable of conjugative transfer, although a 2-log decrease in efficiency was observed when compared to wild-type R27 transfer. The C-terminal GFP moiety may impair the function of the conjugative apparatus; however, the assembly of a cellular machine containing TrhC-GFP must occur to support pDT3048 transfer.

The dependency on transfer proteins other than TrhC-GFP for focus formation signified that the fluorescent foci likely

represent transfer protein assemblies. When TrhC-GFP was produced from the expression construct pMWG191, cells were a confluent green (i.e., no foci were present), but in the presence of both wild-type R27 and pMWG191, foci were again observed (7). This implied that R27-encoded proteins were part of a protein complex containing enough TrhC-GFP molecules to be visible. In addition, when three transposon mutants of R27 (*trhB*, *trhE*, and *trhL* mutants) were present with the *trhC-gfp* expression vector, fluorescent foci were not visible (7), indicating that specific transfer proteins were required for complex assembly.

We previously generated transfer mutants in two separate regions of R27 encoding transfer functions (Tra1 and Tra2) by transposon and site-directed mutagenesis (Table 1), and in this study each of these transfer-deficient mutants was used in conjunction with pMWG191 to evaluate the requirements for TrhC-GFP to enter into membrane-associated protein complexes. Notably, each of the R27 transfer mutants has previously been complemented for transfer by the production in *trans* of the respective wild-type transfer protein (19, 20); therefore, extreme polar effects caused by the gene disruptions were not observed.

After transformation of the *trhC-gfp* expression plasmid pMWG191 separately into *E. coli* cells containing one of the 19 different R27 transfer mutants, preparations of live cells were visualized under UV illumination (Fig. 1). Twelve of the 19 R27 transfer mutants were unable to support the formation of fluorescent foci, as these cells contained, on average, between 0.0 and 0.4 foci per cell, and the percentages of cells containing one focus or more ranged from 0.8 to 15% among the different mutants (Fig. 1). We speculate that in these instances in which a particular mutation prohibits focus formation, the missing transfer protein is a component of the TrhC-GFP-containing complex or is directly required for its assembly. The loss of these requisite components likely results in incomplete (i.e., transfer-deficient) subassemblies that are below the threshold level of visualization.

In cells containing pMWG191 and an R27 transfer mutant of *trhC*, *trhA*, *trhK*, *trhW*, *traG*, *traI*, or *traH* (Fig. 1), fluorescent foci were observed. The average numbers of foci per cell ranged from 1.3 to 4.4, whereas the percentages of cells containing foci ranged from 43 to 93% among these mutants (Fig. 1). These genes, with the exception of *trhC*, are not strictly required for the assembly of fluorescent foci, and in particular, cells lacking TrhW (predicted inner membrane Mpf protein) or TraH (predicted relaxosomal component) demonstrated no reduction in foci. In contrast, intermediary effects on the formation of foci were observed in cells lacking TrhA (pilin subunit), TrhK (predicted outer membrane secretin), TraG (inner membrane coupling protein), and TraI (DNA relaxase). These results suggest that several of the essential conjugative transfer proteins are not required for focus formation. The exact composition of the TrhC-GFP protein complex was not obtained in these experiments, and each of the above six transfer proteins may still be components of the TrhC-containing complex, but they are not absolutely required for its assembly. The intermediate effects seen with four transfer mutants might have been elicited by unknown consequences of each mutation (including the altered stability of other essential transfer proteins).

In the fluorescence assay, TrhC is produced in two forms: TrhC-GFP (from pMWG191) and TrhC (from all R27 transfer mutants except the *trhC* mutant). For determination of the effect of coproduction of the two TrhC species on focus formation, cells carrying pMWG191 and wild-type R27 were examined (Fig. 1). The fluorescence patterns of these cells were similar to those of cells carrying pMWG191 and an R27 *trhC* mutant; therefore, the presence of native TrhC does not affect TrhC-GFP condensation into foci. Furthermore, the overproduction of TrhC-GFP from pMWG191 results in an approximately 2-log unbalanced stoichiometry between the fusion protein and the other transfer proteins (our unpublished observations). The excess TrhC-GFP in cells containing foci was likely seen as the confluent (non-focus-associated) fluorescence, similar to that seen in cells lacking R27 but producing TrhC-GFP.

Generation of NTP-binding motif mutations in *trhC* and *trhC-gfp*. We previously cloned *trhC* into the expression vector pMS119EH to create pMWG36 (Table 1). After site-directed mutagenesis using primers to introduce deletions and substitutions into the Walker A and B motif-encoding regions of *trhC* (pMWG36) and *trhC-gfp* (pMWG191) (Fig. 2), the resulting plasmid constructs were transformed into both DY330R cells and DY330R cells containing pDT2969 (an R27 *trhC* transfer mutant). In the Walker A motif, mutations were introduced in the region encoding Gly-Lys-Ser (GKS). These included the replacement of all three residues with the small, nonpolar amino acid alanine (GKS to AAA), a single Ala substitution of the similarly small, nonpolar Gly (AKS), Gly replaced by a small, polar Ser (SKS), and the more dramatic mutation of Lys (positively charged, polar) to Glu (acidic side chain, polar) (GES). In the Walker B motif, we deleted the Asp-Glu-Ala-Trp (DEAW) subregion and also replaced the highly conserved charged polar residues Asp and Glu (DE subregion) with uncharged Asn (NE) and Glu (EE). We also created a tandem substitution in both the Walker A and B motifs (AAA/EE) by using two successive rounds of primer-mediated site-directed mutagenesis.

Mutations in the NTP-binding motifs of TrhC do not affect fluorescent focus formation. In the fluorescence assay (Fig. 2), the production of TrhC-GFP fusion proteins (the wild type or NTP-binding motif mutants) in cells lacking pDT2969 resulted in confluent, uniform fluorescence. For any of these trials in which the complement of R27-encoded proteins was absent, the average number of foci per cell was $\leq$0.2 and the percentage of cells having one or more foci was $\leq$5.0% (Fig. 2). In contrast, the production of TrhC-GFP (the wild type or NTP-binding motif mutants) within cells containing pDT2969 resulted in an average of $\geq$2.9 foci per cell, with $\geq$91% of cells having one or more foci (Fig. 2). When the extents of foci present in cells producing wild-type TrhC-GFP and in cells producing mutant TrhC-GFP were compared, little difference was observed. These data indicate that the NTP-binding motifs are not required for the assembly of TrhC-associated transfer protein complexes.

Mutations in the NTP-binding motifs of TrhC prohibit plasmid transfer. To determine the effect of NTP-binding motif mutations on conjugative transfer, we conducted standard conjugative transfer complementation experiments. The ability of pMWG36 and its derivatives to restore, in *trans*, the transfer of three different R27 *trhC* transfer mutants was tested (Table 2). The production of wild-type TrhC from pMWG36 restored the

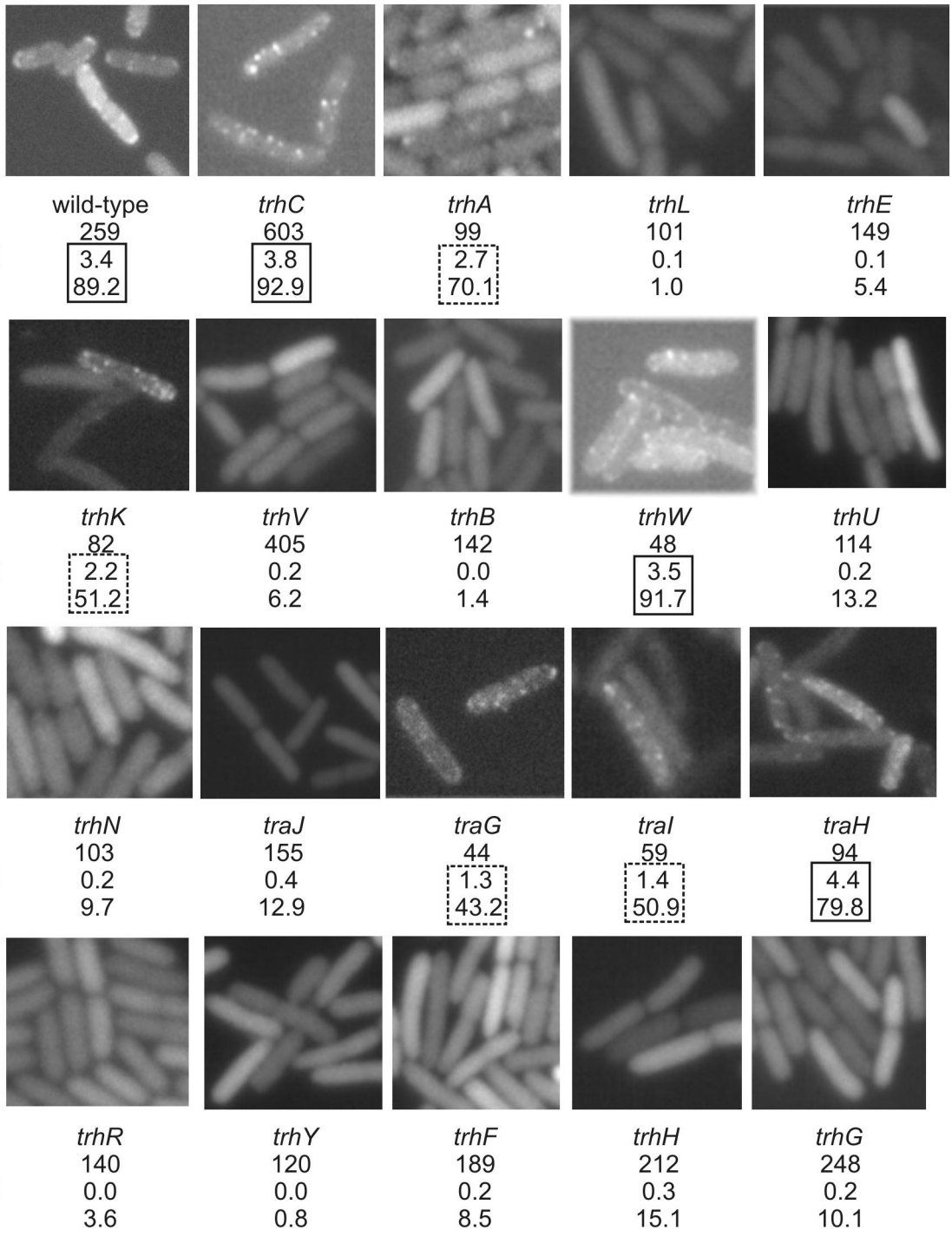

FIG. 1. Fluorescence microscopy of live *E. coli* cells harboring pMWG191 and an R27 transfer mutant. *n* cells were evaluated for the presence of fluorescent foci by criteria that were described previously (7). Solid-line boxes indicate populations of cells with fluorescence patterns similar to that of R27 plus pMWG191, whereas dashed-line boxes indicate mixed populations, in which a large proportion of individual cells have either foci or homogenous fluorescence.

transfer of pDT2957-, pDT2969-, and pDT2978-containing donor cells to wild-type R27 transfer levels ($\sim 10^{-1}$ transconjugant/donor under these conditions) (data not shown). All mutated pMWG36 constructs had no or severely impaired ($>10^6$-fold reduction) complementation of pDT2957- and pDT2978-containing cells (Table 2). The tandem mutation of both the Walker A and B motifs also did not complement transfer, and cumulatively, these results suggest that the NTP-binding motifs of TrhC are required for conjugative DNA transfer. Comparable results were also found during mutational studies of VirB4 from the Ti plasmid (2) and the related TrbE from IncP plasmid RP4 (24).

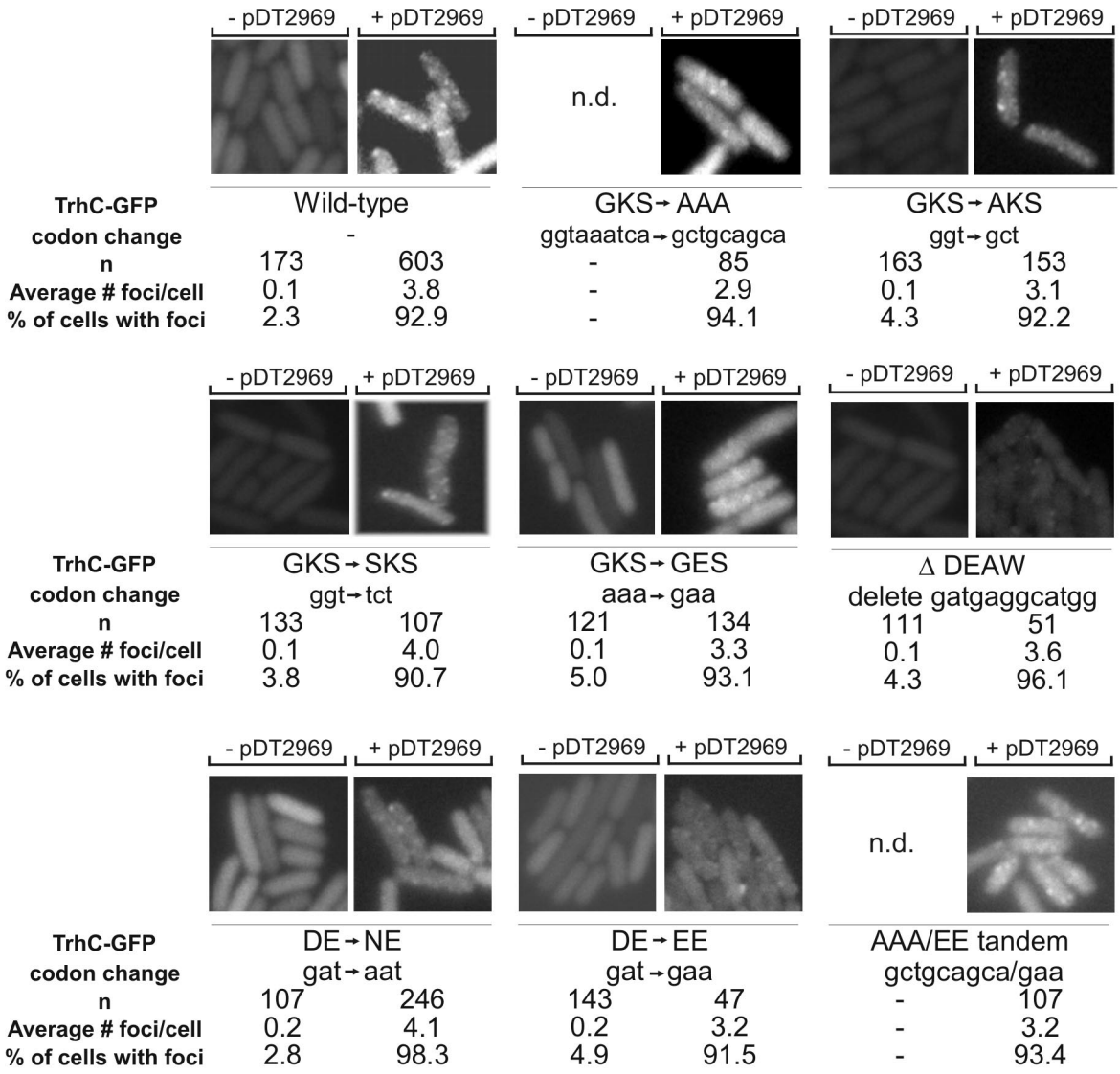

FIG. 2. Fluorescence microscopy of live *E. coli* cells harboring pMWG191 or derivatives encoding mutations in the Walker A or B motif with (right panels) or without (left panels) pDT2969. *n* cells were evaluated for the presence of fluorescent foci by criteria that were described previously (7). n.d., not determined.

The complementation of pDT2969 with Walker A mutation constructs of pMWG36, compared to the complementation results with wild-type pMWG36, resulted in an approximately 10^4-fold reduction in transfer frequency (Table 2). The insertion of mini-Tn*10* into the *trhC* gene in pDT2957 and pDT2978 occurred upstream of the Walker A-encoding region, whereas in pDT2969, mini-Tn*10* was inserted downstream of the Walker A sequence but upstream of the Walker B sequence (32). It is possible that a truncated TrhC peptide containing an unaltered Walker A motif is produced from pDT2969 which can weakly support transfer when full-length, albeit mutated, TrhC is supplied in *trans*. TrhC-like peptides might also be produced from pDT2957 and pDT2978, but considering the point of mini-Tn*10* insertion in these plasmids (32), no peptides containing the Walker A or B motif would likely be produced.

It is also possible that recombination between pMWG36 mutants and pDT2969 occurred, generating a self-transferable form of pDT2969 which resulted in the observed transfer events. To rule out this possibility, we used transconjugants from each pMWG36 mutation construct plus pDT2969 mating event as donors. No transconjugants were detected in this subsequent mating (data not shown), suggesting that transconjugants from the primary mating received intact pDT2969, not a recombinant form containing a functional copy of *trhC*.

Complementation of pDT2969 with Walker B mutation constructs of pMWG36 gave variable results (Table 2). The deletion of amino acids DEAW or the mutation of Asp to Arg did not support transfer, whereas a mutation of Asp to Glu decreased transfer only ~1,000-fold. The capability of the Asp-Glu mutation to partially complement transfer may result from the subtle biochemical difference between these two amino acids.

Mutations in the NTP-binding motifs of TrhC prohibit phage uptake and propagation. Another characteristic we used

TABLE 2. Complementation of conjugative transfer and susceptibility of donor cells containing (i) R27 *trhC* transfer mutants and ii) pMWG36 or derivatives encoding TrhC Walker motif mutations to a bacteriophage

pMWG36 mutant[a]	Conjugative transfer or phage susceptibility with indicated R27 mutant plasmid[b]					
	pDT2957		pDT2978		pDT2969	
	Transfer	Phage sensitive	Transfer	Phage sensitive	Transfer	Phage sensitive
Wild type	8.3×10^{-2}	+	2.3×10^{-1}	+	2.4×10^{-1}	+
AAA	0	−	0	−	9.7×10^{-5}	−
AKS	0	−	0	−	6.0×10^{-5}	−
SKS	0	−	0	−	7.7×10^{-5}	−
GES	0	−	0	−	3.6×10^{-5}	−
Deletion of DEAW	0	−	$<10^{-7}$	−	0	−
NE	0	−	0	−	0	−
EE	$<10^{-7}$	−	$<10^{-7}$	−	1.6×10^{-4}	−
AAA/EE	0	−	0	−	7.2×10^{-7}	−

[a] Underlined residues indicated amino acid substitutions in subregions of the Walker A (GKS) and/or Walker B (DEAW) motif encoded in the pMWG36 derivatives.

[b] Units of transfer are numbers of transconjugants per donor. A transfer frequency of 0 refers to the absence of colonies on plates selecting for transconjugants, whereas $<10^{-7}$ refers to the presence of a small amount of transconjugant colonies that lie outside the dilution range of the experiment to accurately define a transfer frequency. Susceptibility to the H-pilus-specific bacteriophage Hgal was determined in phage spot assays, and lysis was scored as "+," whereas resistance was scored as "−."

to determine the functionality of *trhC* clones encoding NTP-binding motif mutations was the susceptibility of donor cells to a pilus-specific bacteriophage that lyses cells with functional Mpf determinants. By using Hgal, a bacteriophage specific for IncH plasmid-containing cells (22), we were able to determine which donor cells harboring pDT2957, pDT2969, or pDT2978 plus pMWG36 or pMWG36 derivatives were vulnerable to this RNA phage (Table 2). For those cells containing wild-type pMWG36 and any of the three R27 transfer mutants, there was significant lysis, whereas cells containing any of the pMWG36 constructs encoding mutations in the *trhC* Walker motifs and any of the three R27 *trhC* transfer mutants were resistant to Hgal (Table 2). Phage susceptibility depends on the Mpf proteins, and the inability of TrhC NTP-binding motif mutants to support this function (or conjugative transfer) indicates that these are critical domains for the overall functions supplied by TrhC.

Production and stability of TrhC and TrhC-GFP. Mutations made in the Walker A and B motifs of TrhC and TrhC-GFP and the expression of TrhC-GFP in cells containing different complements of transfer proteins could result in an altered stability of TrhC and TrhC-GFP. If this is true, insufficient amounts of TrhC protein may be present to support the complementation of conjugative transfer or the formation of fluorescent foci. For determination of the level of intact TrhC or TrhC-GFP produced in each strain during fluorescence, complementation, and bacteriophage susceptibility assays, whole-cell lysates were probed with an anti-TrhC polyclonal serum (Fig. 3). In all circumstances, either TrhC or TrhC-GFP was detected in cells carrying R27 transfer mutants and pMWG36 or pMWG191. The predominate species detected in each lysate correlated to whole TrhC or TrhC-GFP, and few degradation products were observed. These data suggest that the reduced transfer frequency of R27 when supported by a TrhC Walker A or B mutant resulted from the alteration of the NTP-binding domain rather than from decreased stability of the mutants. Additionally, the stability of TrhC-GFP was not drastically influenced by the absence of individual transfer proteins; therefore, the deficiency of foci produced by 12 R27 transfer mutants is likely due to the necessity of those transfer proteins for protein complex assembly rather than to TrhC-GFP degradation in their absence.

DISCUSSION

The R27 conjugative transfer system encodes 16 Mpf proteins and a coupling protein which are presumed to assemble into a transmembrane apparatus (6, 19, 20). Each of the Mpf proteins is required for plasmid transfer and is predicted to localize to the cell envelope, and all Mpf proteins except TrhN

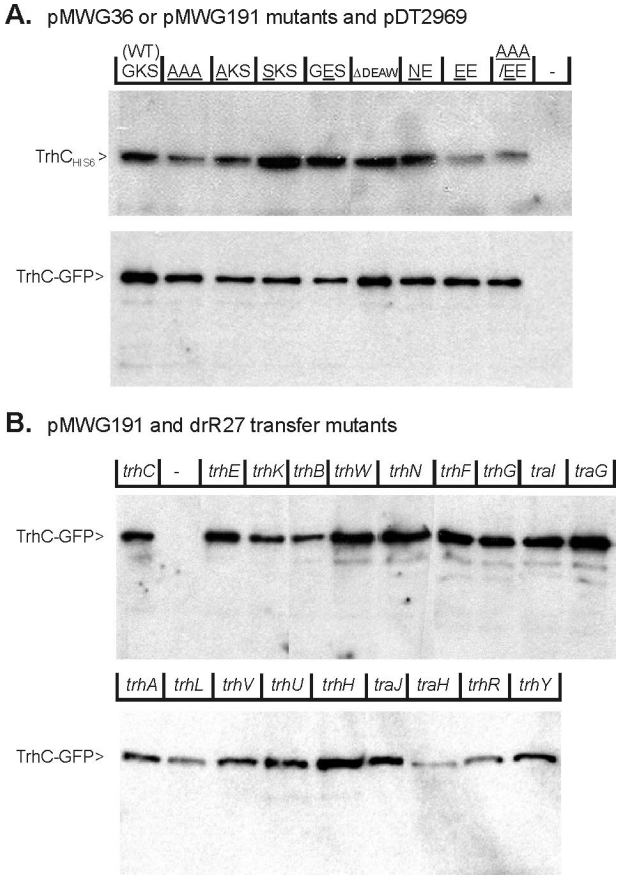

FIG. 3. Production and stability of TrhC and TrhC-GFP. A polyclonal TrhC antiserum (7) was used in an immunoblot to detect TrhC or TrhC-GFP in whole-cell lysates of IPTG-induced cultures harboring wild-type or mutant pMWG36 or pMWG191 plus pDT2969 (A) or pMWG191 plus R27 (B) transfer mutants. Underlined residues indicated amino acid substitutions in subregions of the Walker A (GKS) and/or Walker B (DEAW) motif. −, whole-cell lysate of RG192 plus pDT2969 used as a negative control. Approximately equal amounts of total protein were loaded from each lysate, as the amount of sample used was equalized by using optical density measurements of each culture prior to sample preparation and was verified by Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

and TrhU are required for the production of the conjugative pilus structure (20). Membrane-associated protein complexes minimally comprising the Mpf protein TrhC were previously visualized as discrete foci by use of a TrhC-GFP fusion (7). In this study, we determined the assembly requirements for fluorescent foci by using a library of R27 transfer mutants and by creating mutations in the TrhC NTP-binding motifs.

In our experimental system, GFP foci represent clusters of protein, since a lone GFP molecule would be undetectable as a discrete signal. We predict that foci represent heterologous complexes of transfer proteins composed partly of TrhC-GFP rather than homogenous assemblies of TrhC-GFP molecules. The complex nature of a TrhC-GFP focus was revealed by the dependence on 12 other Trh and Tra proteins for assembly. Eleven of the 12 proteins that are essential for TrhC-GFP focus formation have been classified as Mpf proteins, with the only exception being TraJ. This transfer protein is predicted to have four transmembrane domains by the program TMHMM (16) and is therefore likely to be an inner membrane-associated protein (data not shown). TraJ could possibly interact with other transfer proteins at this cellular location. The protein complex represented as a fluorescent focus may therefore be a subassembly composed of select cell envelope-associated transfer proteins, including both Mpf and non-Mpf proteins.

Distinct protein subcomplexes have also been characterized from the VirB T-DNA transfer apparatus of *Agrobacterium tumefaciens* that are not composed of the entire complement of VirB proteins but are hypothesized to be the core for construction of the remainder of the transfer apparatus (14, 15). These include complexes of VirB6-B8-B9 which can form independently of the other Vir proteins (14) and a VirB7-B9-B9-B10 complex that was extracted from the membrane of *A. tumefaciens* (15). VirB8 was observed to be essential for the assembly of VirB9- and VirB10-associated protein complexes (17). There is no evidence that the 12 transfer proteins required for the formation of TrhC-GFP foci participate in direct interactions with TrhC. Instead, these proteins may cumulatively represent a network of interacting proteins of which TrhC is a member. The assembly of this network of proteins may be ordered, and the loss of an initializing component would likely prevent complete assembly, observed in this study as the absence of fluorescent foci. Furthermore, TraA, TraK, TraG, and TraI are each not required for focus formation, but their absence caused a 25 to 53% reduction in the number of cells containing foci. These proteins may be part of the interaction network but may play an auxiliary role in complex assembly and are not absolutely required. The R27 type IV secretion coupling protein TraG has been demonstrated to interact with the Mpf protein TrhB (6), and this may account for the observed influence of TraG on TrhC-associated complex assembly, of which TrhB is a member. The TraG-TrhB interaction and the influence of TraG on the TrhC-containing complex highlight the relationship of the different structures that compose the transfer apparatus: distinct subassemblies and complexes may be observed, but the coordination of all of the elements as a singular unit may ultimately be required for its functioning.

The precise functional role of TrhC (and TrhC NTP-binding motifs) in the transfer apparatus is undetermined. Our results suggest that the functioning of the transfer system (including DNA transfer and pilus production) is dependent on the NTP-binding motifs of TrhC but that the assembly of the apparatus is not. We demonstrated that numerous mutations affecting the TrhC NTP-binding domains had little effect on the formation of the TrhC-associated subassembly of transfer proteins. It has also been suggested that the assembly of VirB protein subcomplexes does not require NTP binding or metabolism by VirB4 (4). VirB4 Walker A motif mutants did not diminish the stimulated uptake of plasmid RSF1010 in recipient cells producing a subset of the VirB proteins, including the VirB4 mutants (4). Although NTPase activity has not been demonstrated for TrhC or any related proteins in vitro, the in vivo conditions may support this energy-providing function for the translocation of DNA and/or pilin substrates.

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