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Outer Membrane Components of the Tad (Tight Adherence) Secreton of *Aggregatibacter actinomycetemcomitans*▽†

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Prokaryotic secretion relies on proteins that are widely conserved, including NTPases and secretins, and on proteins that are system specific. The Tad secretion system in *Aggregatibacter actinomycetemcomitans* is dedicated to the assembly and export of Flp pili, which are needed for tight adherence. Consistent with predictions that RcpA forms the multimeric outer membrane secretion channel (secretin) of the Flp pilus biogenesis apparatus, we observed the RcpA protein in multimers that were stable in the presence of detergent and found that *rcpA* and its closely related homologs form a novel and distinct subfamily within a well-supported gene phylogeny of the entire secretin gene superfamily. We also found that *rcpA*-like genes were always linked to *Aggregatibacter rcpB*- or *Caulobacter cpaD*-like genes. Using antisera, we determined the localization and gross abundances of conserved (RcpA and TadC) and unique (RcpB, RcpC, and TadD) Tad proteins. The three Rcp proteins (RcpA, RcpB, and RcpC) and TadD, a putative lipoprotein, localized to the bacterial outer membrane. RcpA, RcpC, and TadD were also found in the inner membrane, while TadC localized exclusively to the inner membrane. The RcpA secretin was necessary for wild-type abundances of RcpB and RcpC, and TadC was required for normal levels of all three Rcp proteins. TadC abundance defects were observed in *rcpA* and *rcpC* mutants. TadD production was essential for wild-type RcpA and RcpB abundances, and RcpA did not multimerize or localize to the outer membrane without the expression of TadD. These data indicate that membrane proteins TadC and TadD may influence the assembly, transport, and/or function of individual outer membrane Rcp proteins.

Aggregatibacter (formerly *Actinobacillus*) *actinomycetemcomitans* is a gram-negative, capnophilic coccobacillus, principally known as the etiologic agent of localized aggressive periodontitis (16, 25, 39). *A. actinomycetemcomitans* is occasionally able to colonize sites outside of the oral cavity to produce other infections, and it is one of the so-called HACEK organisms, gram-negative bacteria that cause approximately 3% of infective endocarditis cases (20). Clinical isolates of *A. actinomycetemcomitans* adhere to surfaces nonspecifically and form remarkably strong biofilms (14, 15, 29). Insertional mutagenesis studies have shown that genes of the *tad* (*t*ight *ad*herence) locus are necessary for the nonspecific adherence of the bacterium to surfaces, as well as for the phenotypes of rough colony morphology, autoaggregation, and production of type IVb Flp pili (29, 31, 42, 45). Genetic and biochemical analyses have indicated that 13 *tad* gene products are involved (3, 29, 31, 42, 45, 66); only the *flp-2* product is not (42).

tad loci have been identified in over half of the sequenced bacteria and in all of the archaeal genomes that have been completed (45, 52). Phylogenetic evidence strongly indicates that many bacterial species have acquired the *tad* genes from foreign sources, and because of its apparent propensity for horizontal transfer, the *tad* locus has also been named the widespread colonization island (44, 45). The *tad* locus has been implicated in the pathogenesis of several bacterial diseases. *A. actinomycetemcomitans* strains with inactivated *flp-1* or *tadA* fail to colonize tooth surfaces and cause characteristic bone loss, reminiscent of localized aggressive periodontitis, in a rat model of that disease (59). A *tadA* mutant of the human pathogen *Haemophilus ducreyi* is avirulent in a skin pustule test (62). During in vivo assays to identify virulence genes, *tadD* was identified as likely to be critical for virulence in both the animal pathogen *Pasteurella multocida* and the fish-colonizing bacterium *Yersinia ruckeri* (12, 17). Thus, the study of *tad* loci and the molecular functions of Tad proteins is predicted to produce valuable insights about the life styles of diverse and disease-causing prokaryotes.

Many *tad* locus proteins (Flp1, TadV, RcpA, TadA, TadB, TadC, TadE, and TadF) exhibit similarity to proteins that participate in type II secretion (T2S), type IV pilus (T4P) assembly, type IV secretion, and type III secretion (T3S), but some of the Tad proteins (RcpB, RcpC, TadZ, TadD, and TadG) are not significantly similar to known proteins. The Tad proteins are predicted to assemble to form a macromolecular structure for the assembly and secretion of Flp pili (67). Although the *tad* genes are widespread, the architecture and operation of the Tad secretion system have not been elucidated.

*R*ough-*c*olony *p*roteins RcpA and RcpB were discovered in an outer membrane fraction from adherent, rough-colony-forming *A. actinomycetemcomitans* cells, and three open reading frames, called *rcpCAB*, were found by nucleotide sequencing (23). We mapped the *rcp* genes to the *tad* locus (see Fig.

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1A) (29). As in other genes of the *tad* locus, transposon insertions in *rcpA* and *rcpC* abolish the nonspecific adherence and associated phenotypes, including Flp-fibril secretion, and these defects can be restored by genetic complementation (45). Our attempts to inactivate *rcpB* in adherent strains failed to produce viable transformants, and we determined that *rcpB* is essential for viability in the context of an intact *tad* locus (42).

Based on its sequence and its presence in the outer membrane, the RcpA protein is expected to form the channel through which Flp pili of the Tad secretion system traverse the outer membrane (23). The RcpA homolog CpaC has been localized to the pole of the environmental organism *Caulobacter crescentus*, whose *tad* locus has been named *cpa* for C*aulobacter p*ilus *a*ssembly (60). Both CpaC and RcpA contain C-terminal domains that are conserved among known secretin proteins (see Fig. 1B), including the proteins PulD of *Klebsiella* spp., pIV of filamentous phage, and PilQ of *Neisseria gonorrhoeae* (19, 53, 60). The evolutionary relationship of the RcpA protein to other secretins and its ability to function as a secretin are not well-characterized.

Although *rcpB* is probably essential for the production of Flp pili, an outer membrane function for RcpB cannot be inferred from its predicted primary sequence. Obvious homologs of *rcpB* are found exclusively in *tad* loci of members of the *Pasteurellaceae* family (30). The *cpaD* gene, located in the *cpa* locus in a position analogous to that of *rcpB* (i.e., between homologs of *rcpA* and *tadZ*), may be a relative of *rcpB* despite our inability to detect any sequence similarity, and *cpaD* is known to be required for pilus biogenesis in *C. crescentus* (60).

Sequence predictions indicate that RcpC and TadD may localize to the bacterial outer membrane. The RcpC protein does not have a strongly predicted function. *tadD* is absent from the *tad* loci of gram-positive bacteria, as is true for the *rcpAB* genes, which encode proteins known to localize to the outer membrane (67). The protein sequence of TadD contains tetratricopeptide repeat (TPR) domains and a predicted site for N-terminal cleavage by signal peptidase II. These sequence features are common among a class of known outer membrane lipoproteins, called docking proteins for their known role in positioning secretin proteins at the outer membrane (2, 6, 40). Finally, TadC displays similarity to PilC-like proteins (41) and is likely to have multiple inner membrane-spanning segments, which presents the likelihood that this protein may be required for the stability of other members of the Tad system, potentially as an inner membrane scaffold. The localization and characterization of these proteins in *A. actinomycetemcomitans* have not been reported.

In this study, we examined properties of known and predicted membrane proteins of the Tad secretion system. We have compared the RcpA amino acid sequence to sequences of other secretins to demonstrate the evolutionary relationship of RcpA within the protein superfamily and among *tad* loci-containing organisms. We also generated antisera specific to Rcp and Tad proteins to demonstrate evidence of RcpA multimer formation and to show the localization patterns of several Tad proteins and the Tad-related requirements for their presence.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The addition of "N" to a strain designation denotes a spontaneous nalidixic acid-resistant derivative. Two derivatives of rough clinical isolates, CU1000N (serotype *f*) (29) and DF2200N (serotype *a*) (32), were used as wild-type strains. *A. actinomycetemcomitans* strains were grown and stored in *A. actinomycetemcomitans* growth medium (AAGM) broth or agar, each with 0.75% glucose (wt/vol) and 0.4% sodium bicarbonate (wt/vol), as previously described (65). Chloramphenicol (2 μg/ml) and nalidixic acid (20 μg/ml) were used to supplement AAGM agar plates or broth, as necessary.

Escherichia coli Top10 (Invitrogen) was used for all subcloning procedures and served as a conjugative donor for the mobilization of broad-host-range IncQ expression vectors into *A. actinomycetemcomitans*. As described previously (21, 65), an *ori*T-defective mutant of the IncP RK2 plasmid, pRK21761, which does not self-transfer efficiently, was used to mobilize IncQ vectors, including pJAK17 (J. A. Kornacki, unpublished data). *E. coli* strains were grown on Luria-Bertani (LB) (56) agar plates or in LB broth at 37°C, and kanamycin (50 μg/ml) or chloramphenicol (50 μg/ml) was used for the appropriate selection of plasmids. Electroporation or $CaCl_2$ transformation of *E. coli* with recombinant plasmids was done as described previously (7). For the expression of all proteins in *E. coli* strain BL21(DE3), LB was supplemented with the Overnight Express autoinduction system (Novagen) and the cells were grown at 30°C.

DNA manipulations. All *A. actinomycetemcomitans* gene sequences were amplified from CU1000N genomic DNA that was extracted using the DNeasy tissue kit (QIAGEN). PCR was done using the high-fidelity DNA polymerase TripleMaster *Taq* (Eppendorf) and primers that were purchased from Sigma Genosys. Restriction endonucleases and T4 DNA ligase were used according to the instructions of the manufacturer (New England Biolabs). After agarose gel electrophoresis, the extraction of digested plasmid or amplified DNA was done using either the QIAex II kit (QIAGEN) or SpinX tubes (Corning). Extracted PCR products were ligated into the pCR2.1-TOPO TA cloning vector (Invitrogen). Plasmid preparations were made with the QIAprep spin miniprep kit (QIAGEN), and all cloned PCR products were confirmed by nucleotide sequencing at the Columbia University DNA sequencing facility by using an Applied Biosystems 3100 capillary sequencer.

Protein sequence analysis. Within the MacVector 7.2 software (Accelrys Inc.), the ClustalW algorithm was used to produce amino acid alignments. The NCBI Entrez (http://www.ncbi.nlm.nih.gov) sequences used were those of *A. actinomycetemcomitans* RcpA (AAN75208), *C. crescentus* CpaC (AAF40192), and *Klebsiella pneumoniae* PulD (AAA25126.2). PSORT (http://www.psort.org) (18) and CELLO (http://cello.life.nctu.edu.tw/) (70) were used to make predictions of protein features and subcellular localization in gram-negative bacteria.

Database sequence collection and phylogenetic analysis. Sequences of the following proteins were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov) and used for searching for the secretin superfamily: RcpA from *A. actinomycetemcomitans*, CpaC from *C. crescentus*, PilQ from *Pseudomonas aeruginosa*, PulD from *Klebsiella pneumoniae*, InvG from *Salmonella enterica*, SpiA from *S. enterica*, BfpB from *E. coli*, TcpC from *Vibrio cholerae*, HrcC from *Erwinia amylovora*, and pIV from filamentous phage from *E. coli*. Each sequence was used in a separate BLAST search (1) of GenBank and unfinished genome sequences available in the NCBI database, and the results from each search were compiled. Default settings of the BLAST program were used, except that we did not use a low-complexity filter, as this option often excluded clearly conserved regions from similarity calculations. In all, a nonredundant data set of 186 amino acid sequences was used, each of which significantly exceeded BLAST default criteria.

Protein sequences were aligned using CLUSTAL X 1.63 (64), gap parameters were varied in three independent alignments, and the three resulting matrices were concatenated for analysis: gap/change ratios for each alignment were 4, 7, and 10. This method is called elision and can be used to account for the possibility that certain portions of the alignment are incorrect (68). The alignment is available upon request.

We did parsimony analysis using the heuristic "ratchet" method (38), implemented with the aid of PAUPRat (Sikes & Lewis, Storrs, CT) in conjunction with PAUP. Two hundred replicates of the ratchet method were done. In each iteration, 15% of the characters were upweighted using tree-branch reconnection (TBR). Only one tree at each step was saved. TBR with the MulTrees option was done on the resulting trees. All characters and state transformations were given equal weight, and columns with gaps were retained as phylogenetically informative characters (43).

To calculate confidence in the trees, we generated bootstrap values and Bre-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
A. actinomycetemcomitans		
Aa0727	Aa1577 with pJMM108; Km^r Nal^r Cm^r	Morrison and Figurski, unpublished
Aa0886	CU1000N *tadZ*::IS*903*ϕ*kan*; Km^r Nal^r	45
Aa1005	CU1000N; spontaneous Nal^r mutant of rough clinical isolate CU1000, serotype *f*; Nal^r	29
Aa1332	CU1000N *tadB*::IS*903*ϕ*kan*; Km^r Nal^r	29
Aa1347	CU1000N *tadE*::IS*903*ϕ*kan*; Km^r Nal^r	29
Aa1354	CU1000N *rcpA*::IS*903*ϕ*kan*; Km^r Nal^r	45
Aa1359	CU1000N *tadC*::IS*903*ϕ*kan*; Km^r Nal^r	29
Aa1360	CU1000N *tadA*::IS*903*ϕ*kan*; Km^r Nal^r	29
Aa1361	CU1000N *rcpC*::IS*903*ϕ*kan*; Km^r Nal^r	45
Aa1511	CU1000N *tadF*::IS*903*ϕ*kan*; Km^r Nal^r	29
Aa1561	CU1000N *tadG*::IS*903*ϕ*kan*; Km^r Nal^r	29
Aa1577	CU1000N *tadD*::IS*903*ϕ*kan*; Km^r Nal^r	29
Aa1758	Aa1577 with pJAK17; Km^r Nal^r Cm^r	This study
Aa1759	Aa1577 with pPP40; Km^r Nal^r Cm^r	This study
Aa3074	DF2200N *tadV*::mini-Tn*5*Km; Km^r Nal^r	42
JK1010	CU1000N *flp-1*::IS*903*ϕ*kan*; Km^r Nal^r	31
MB1228	DF2200N; spontaneous Nal^r mutant of rough clinical isolate DF2200, serotype *a*; Nal^r	32
E. coli		
BL21(DE3)	F^- *dcm ompT hsdS*$_B$(r_B^- m_B^-) *gal* (λDE3)	Novagen
SC1031	BL21(DE3) with pSAC117; Km^r	This study
SC1032	BL21(DE3) with pSAC118; Km^r	This study
SC1041	BL21(DE3) with pPP40; Cm^r	This study
Top10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *deoR* Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG* (ϕ80*lacZ*Δ*M15*)	Invitrogen
Plasmids		
pBP100	RcpB in pCR2.1-TOPO	This study
pBP111	RcpC in pCR2.1-TOPO	This study
pCR2.1-TOPO	TA cloning vector; Ap^r Km^r	Invitrogen
pET30a	Cloning vector used to produce N-terminal fusions to six-His tag; Km^r	Novagen
pJAK17	Broad-host-range mobilizable IncQ vector derived from pMMB67; carries *tac* promoter; Cm^r *lacI*^{q}	Kornacki, unpublished
pJMM108	*tadC* upstream region-*tadD*-T7 coding sequence in pJAK17; Cm^r	Morrison and Figurski, unpublished
pPP40	*rcpA* from CU1000N in pJAK17; Cm^r	45
pSAC117	*rcpC* from CU1000N in pET30a with 5′ BamHI and 3′ EcoRI ends; Km^r	This study
pSAC118	*rcpB* from CU1000N in pET30a with 5′ BamHI and 3′ SalI ends; Km^r	This study

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Nal^r, nalidixic acid resistance; Str^r, streptomycin resistance.

mer decay indices using the program AutoDecay with PAUP (10). Ten TBR replicates at each node in the phylogeny were done to obtain the Bremer index. Because Bremer indices were calculated using the elided data set of three independent alignments, each value was divided by three and rounded to the nearest 10th. We did 100 bootstrap replicates in PAUP, with one iteration of random addition followed by TBR, limiting the search time to 1 h for each replicate. Bootstrap values were retained if they were consistent with the 50% majority consensus tree.

Generation of antisera. RcpC and RcpB recombinant proteins were constructed as follows. The full-length *rcpC* and *rcpB* genes were amplified using the primer sets RcpCBamHIup (5′-**GGATCC**ATGAATTACAGAACGCTCTTG-3′) and RcpCEcoRIdwn (5′-**GAATTC**TTATTGACCTCTTAATTTTCTGATG AA-3′) and RcpBBamHIup (5′-**GGATCC**ATGAGAAAATTAGTTATTACGG CCTC-3′) and RcpBSalIdwn (5′**GTCGAC**TTAATACTTCAATTGAACACGC TGATT-3′), respectively, and then cloned into pCR2.1-TOPO (Invitrogen) to generate pBP111 and pBP100. The RcpC coding sequence was excised with BamHI and EcoRI, and the fragment encoding RcpB was removed with BamHI and SalI. Each of these fragments was then ligated into the expression vector pET30a (Novagen), proximal to a sequence encoding a His_6 tag, producing pSAC117 and pSAC118.

In *E. coli*, His-tagged RcpB and RcpC recombinant proteins were expressed from pSAC117 and pSAC118, whereas the native (full-length) RcpA was expressed from pPP40 (45). Autoinduced cells were treated as described in the protocol for the Bugbuster protein extraction reagent (Novagen), with the addition of 100 mM NaCl and 5 mM $MgCl_2$ to either Bugbuster or the equivalent B-PER II solution (Pierce) to increase protein stability.

Following lysis, the resuspension of insoluble material with 6 M urea solubilized recombinant RcpB and RcpC, each of which was purified on a column (Pierce) by using the His-Bind kit (Novagen) with the addition of 6 M urea to all buffers. Purified proteins were sent to Invitrogen for the immunization of rabbits.

For purification and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of RcpA, insoluble material was resuspended in Laemmli buffer (35) and boiled after Bugbuster lysis. The abundant protein band was

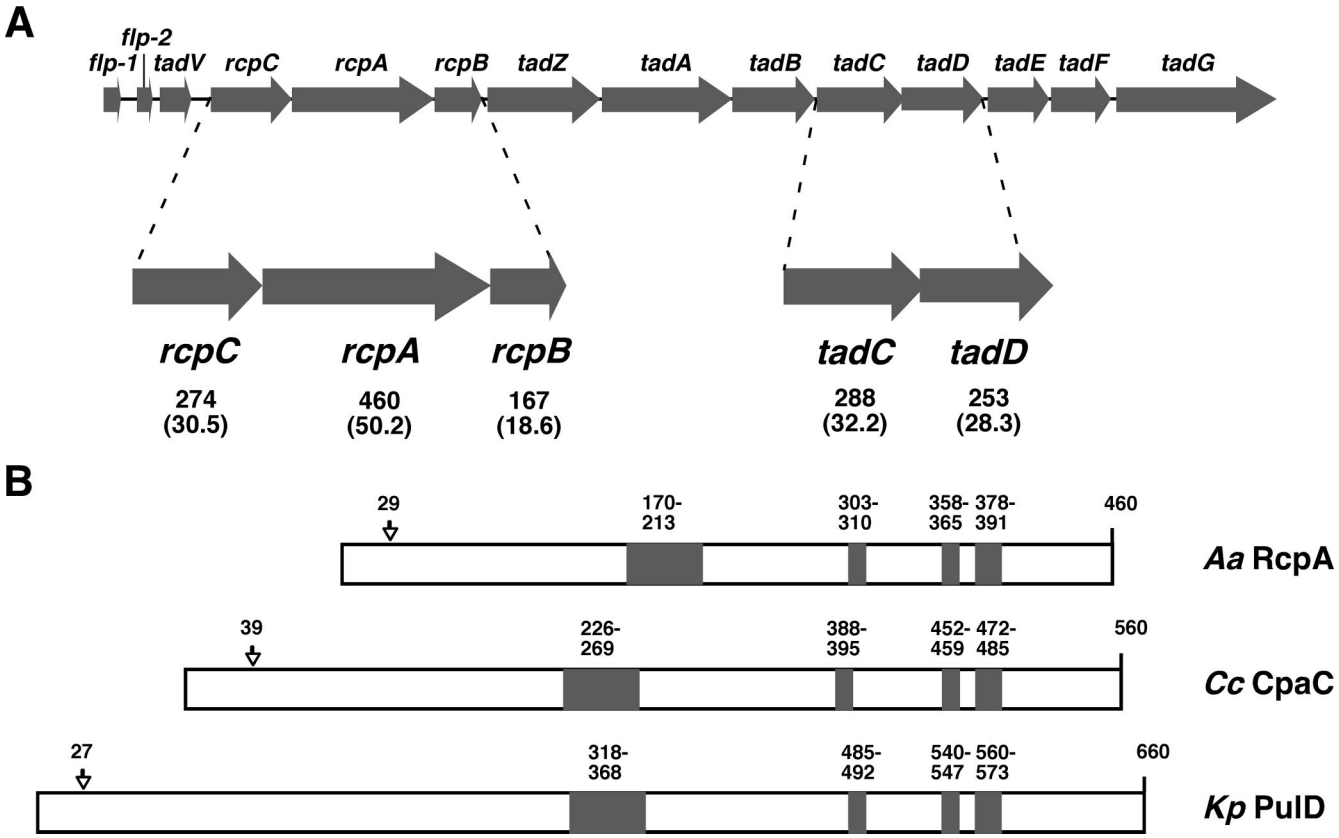

FIG. 1. *tad* locus and conserved secretin sequences. (A) The *tad* locus is composed of 14 genes that are transcribed in the same direction (22). The number of amino acid residues for the full-length protein products of *rcpCAB* and *tadCD* are shown by the top numbers under the genes. The sizes (in kilodaltons) of the proteins are specified by the numbers in parentheses directly below. The nucleotide sequence was from the strain CU1000N. (B) Conservation in secretins. Conserved regions A to D in *A. actinomycetemcomitans* (*Aa*) RcpA, *C. crescentus* (*Cc*) CpaC, and *K. pneumoniae* (*Kp*) PulD are shaded. The residue numbers spanned by each region are denoted. The alignment of the proteins is based on regions C and D, which are spaced equally in all three proteins. Arrows indicate predicted signal sequence cleavage sites.

removed in gel slices and sent for the production of antisera in rabbits (at Cocalico Biologicals, Reamstown, PA).

When necessary, the adsorption of nonspecific antibodies with acetone powders was used to reduce the background (24).

The TadC antiserum was generated using the TadC peptide comprising amino acids 40 to 58 (ERPKDQDSDEVAKNKSKQQ) conjugated to the keyhole limpet hemocyanin carrier protein via an N-terminal cysteine residue. The resulting protein was used to generate specific antisera in rabbits at Zymed Laboratories, Inc. The antisera were enriched with TadC peptide-specific antibodies by affinity purification at Zymed Laboratories, Inc.

Preparation of soluble and membrane fractions. The fractionation of *A. actinomycetemcomitans* strains by differential detergent solubilization was done essentially as described before (3, 23), with a few modifications. Briefly, adherent *A. actinomycetemcomitans* cells were grown in 750-ml tissue culture flasks (BD Falcon), scraped, and pelleted by centrifugation. Nonadherent cells were grown in and harvested from 50-ml conical tubes (BD Falcon). For lysis, the cell pellets of adherent or nonadherent cultures were first frozen and then resuspended in 10 mM HEPES (pH 7.5) with 50 mM NaCl and incubated with 0.1 mg of lysozyme (Sigma)/ml on ice prior to physical disruption by sonication. Sonicates were cleared twice by centrifugation at 7,000 × *g* for 10 min. Subsequent centrifugations were carried out in a type 70.1 Ti Beckman ultracentrifuge rotor at 105,000 × *g* for 1 h. The soluble fraction contained cytoplasmic and, presumably, periplasmic proteins. The pellet, comprising bacterial membranes, was resuspended in buffer containing 0.5% Sarkosyl (*N*-laurylsarcosine; Sigma), and the suspension was rocked for 30 min at room temperature. The Sarkosyl-soluble fraction contained inner membrane proteins, whereas the pellet, resuspended in a 500-μl solution of 10 mM HEPES (pH 7.5) with 50 mM NaCl, 1% Sarkosyl, and 10 mM EDTA, consisted of outer membrane proteins.

For experiments using the strain Aa1759, fractions were prepared from twice the volume of overnight cultures.

Immunoblot analysis. *A. actinomycetemcomitans* strains were grown in 10 ml of AAGM broth for 17 h. Cultures were centrifuged to harvest bacterial cells, and the pellets were resuspended and boiled for 5 min in standard Laemmli loading buffer (35) containing 2% SDS and 5% β-mercaptoethanol. Equal amounts of protein from each strain were resolved by SDS-PAGE and analyzed by immunoblotting with anti-RcpA (1:10,000), anti-RcpB (1:2,500), anti-RcpC (1:10,000), or anti-TadC (1:1,000) antiserum. An anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (HRP; Pierce) was used as a secondary antibody at 1:50,000. We were unable to raise a suitable antiserum for TadD, but for the detection of TadD, we used a functional, tagged version of TadD (TadD-T7) expressed from pJMM108 (J. M. Morrison and D. H. Figurski, unpublished data) and anti-T7 epitope tag antibody (1:5,000; Novagen), along with HRP-conjugated anti-mouse immunoglobulin G secondary antibody at a dilution of 1:50,000 (Pierce). Femto Western chemiluminescence reagent (Pierce) was used as a substrate for HRP.

To determine subcellular localizations by immunoblotting, ethanol precipitation was used to concentrate inner membrane fractions. Protein samples from soluble and membrane fractions were prepared by adding 4× Laemmli loading buffer and were treated as described above.

Multimerization samples were run on 8% polyacrylamide gels after resuspended pellets were heated at 37, 65, or 100°C for 5 min. For the efficient detection of these samples by immunoblotting, it was necessary to use modified electroblot buffer prepared with 10% methanol (71) and to transfer proteins onto nitrocellulose overnight at 30 V.

For experiments using the strain Aa1759, multimerization samples were prepared from twice the volume of overnight cultures.

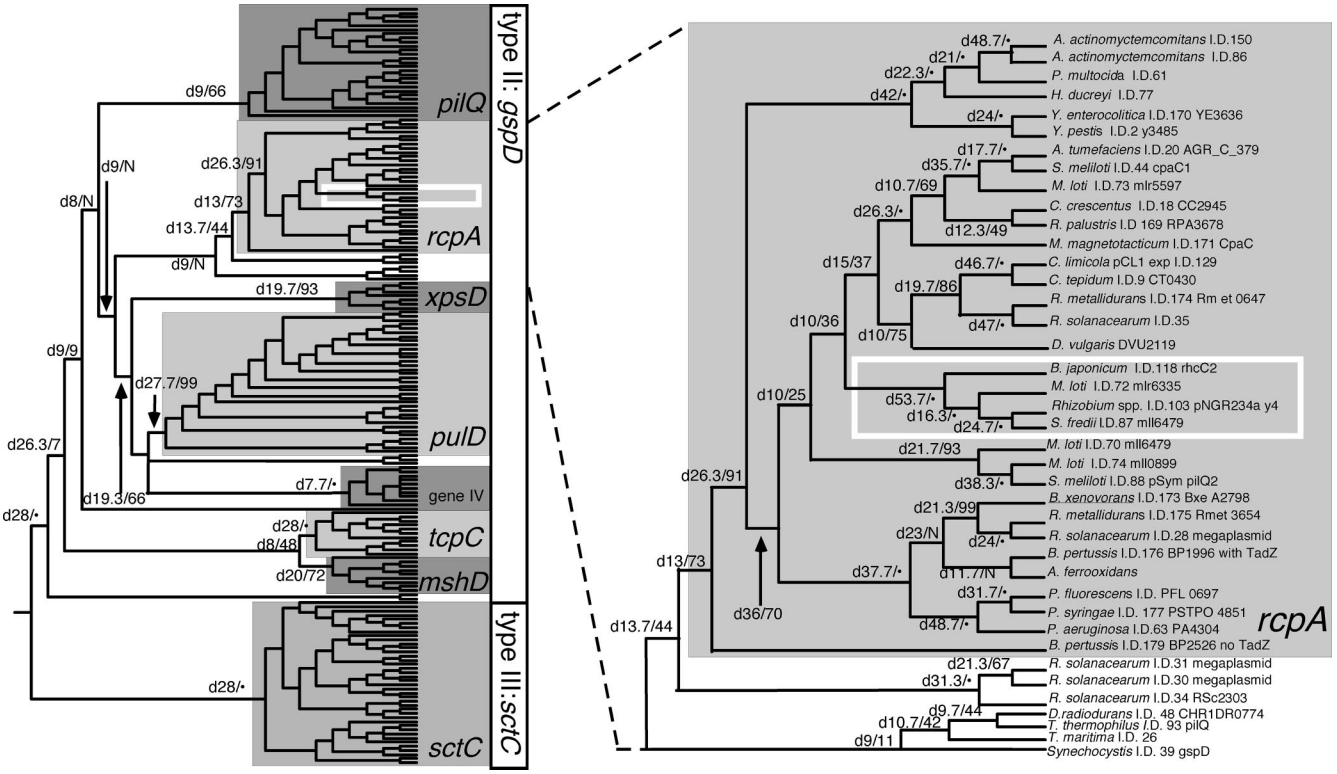

FIG. 2. Phylogeny of secretin gene superfamily. We have divided the secretin gene superfamily into two primary families (*sctC* and *gspD*), whose names are based on proposals for inclusive nomenclature schemes. T3S secretin genes and genes for secretins associated with flagellar biosynthesis are labeled *sctC*, for secretion and cellular translocation, as proposed by Hueck (26). Genes involved in T2S, T4P biosynthesis, and Tad secretion are collectively labeled *gspD*, for general secretion pathway (48, 49). Subfamilies are named for the first described representative of the clade based on our literature review (see the text). The *rcpA* subfamily, along with its closest relatives, is shown enlarged. Within the *rcpA* subfamily, a white box indicates *rcpA* genes that were identified as being adjacent to a putative homolog of *cpaD* without other *tad* locus genes found in the near vicinity. All other members of the *rcpA* subfamily were found with closely linked *tad* locus homologs. Numbers on branches indicate Bremer support values (designated with a "d" by convention) and bootstrap values (a dot indicates a bootstrap value of 100). An expanded superfamily tree with named secretin proteins, a list of secretin genes used, and Bremer and bootstrap indices are available as supplemental figures (see the supplemental material). Complete names of organisms listed are as follows: *Pasteurella multocida*, *Haemophilus ducreyi*, *Yersinia enterocolitica*, *Yersinia pestis*, *Agrobacterium tumefaciens*, *Sinorhizobium meliloti*, *Mesorhizobium loti*, *Caulobacter crescentus*, *Rhodopseudomonas palustris*, *Magnetospirillum magnetotacticum*, *Chlorobium limicola*, *Chlorobium tepidum*, *Ralstonia metallidurans*, *Ralstonia solanacearum*, *Desulfovibrio vulgaris*, *Bradyrhizobium japonicum*, *Sinorhizobium fredii*, *Burkholderia xenovorans*, *Bordetella pertussis*, *Acidithiobacillus ferrooxidans*, *Pseudomonas fluorescens*, *Pseudomonas syringae*, *Pseudomonas aeruginosa*, *Deinococcus radiodurans*, *Thermus thermophilus*, and *Thermotoga maritima*.

RESULTS

***rcpA*-like secretin genes are phylogenetically distinct.** Based on strong scores in BLAST searches and apparently conserved sequence features (Fig. 1B) (23), *rcpA* is a member of the secretin gene superfamily. To examine further the relationship of *rcpA* and its close homologs to other members of the secretin gene family, we undertook a rigorous phylogenetic analysis of the entire superfamily. One hundred eighty-six amino acid sequences were identified, all from organisms in the domain *Bacteria*. No significant homologs in members of the *Archaea* or *Eukarya* or in other organisms with a single lipid membrane were identified, strengthening the idea that secretins are dedicated exclusively to trafficking proteins across the outer membrane.

The secretin superfamily tree was tentatively rooted with the monophyletic clade representing secretins involved in the macromolecular process of T3S (e.g., the product of *sctC*), and secretin genes were divided into two major families (Fig. 2). The *sctC* family, labeled based on the inclusive nomenclature of Hueck (26), includes genes involved in T3S and flagellar biosynthesis. The second family, *gspD*, is based on the inclusive nomenclature of Pugsley and Possot (48, 49), and the *gspD*/T2S subfamilies were named using the first described representative of each clade based on our literature review (5, 33, 54, 55, 61).

rcpA-like genes form a monophyletic clade distinct from genes for secretins for classical T2S (*pulD*), T4P biogenesis (*pilQ*), filamentous phage extrusion (gene IV), and T3S (*sctC*). The *rcpA* subfamily is most closely related to a small clade of putative secretin genes from the *Ralstonia solanacearum* chromosome (RSc2303, accession no. NP_520424) and megaplasmid (RSp0143, accession no. NP_521704 and RSp0474, accession no. NP_522035) (Fig. 2), which are not classified as part of a *tad* locus based on the phylogenetic relationships of nearby genes. Interestingly, a small clade of genes from the *Rhizobiaceae* (e.g., *rhcC2*) is included within the *rcpA* subfamily, but these genes are not found closely linked to *tad* loci or to any other definable loci

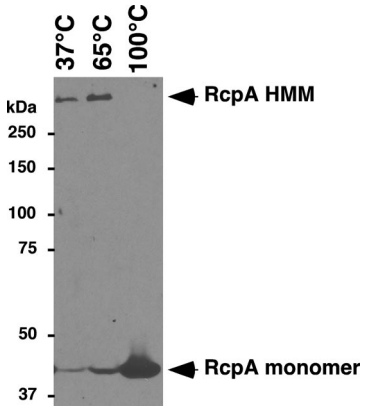

FIG. 3. Multimerization of RcpA. Whole-cell extracts of *A. actinomycetemcomitans* CU1000N (wild type) were heated at 37, 65, or 100°C for 5 min in standard loading buffer and separated by SDS-PAGE through an 8% polyacrylamide gel, as described in the text. Immunoblotting was done with anti-RcpA antiserum. Arrows indicate the positions of the RcpA monomer and the HMM.

for transport systems (Fig. 2). However, in all cases, these genes are found adjacent to a gene with strong similarity to *cpaD*, a gene that is found in the same position as *rcpB* in many *tad* loci. Indeed, to our knowledge, all genes in the *rcpA* subfamily are adjacent to a clear homolog of either *cpaD* or *rcpB*.

Genes for secretins from bacteria outside of the proteobacteria, such as *Thermotoga maritima*, *Deinococcus radiodurans*, *Synechocystis* spp., *Aquifex aeolicus*, and *Fusobacterium nucleatum*, tended to be found at basal (early-branching) positions, suggesting ancient divergences of these genes—reflective of the ancient divergence of the genomes of these bacteria from the proteobacterial lineage. One small clade of secretins from these early-branching organisms is the sister group of the clade composed of the *rcpA* subfamily and the *R. solanacearum* secretin genes discussed above. This phylogenetic pattern indicates that the *rcpA* secretin subfamily has a very ancient origin.

RcpA forms multimers that are stable in the presence of detergent. Known secretin proteins form multimers of 12 to 14 subunits (2, 53, 63) that run as high-molecular-weight species on polyacrylamide gels and resist dissociation by detergent and heat (36, 37). Whole-cell extracts of *A. actinomycetemcomitans* were heated at three different temperatures and examined for multimers by immunoblot analysis with antiserum specific to the RcpA protein. At 37 and 65°C, we detected a band migrating above 250 kDa (Fig. 3). The SDS-resistant high-molecular-weight multimer (HMM) in these samples was accompanied by a minor band at 46 kDa, the correct molecular mass for the monomeric form of RcpA. At 100°C, we detected the lower RcpA band as the dominant species, and no HMM was detected (Fig. 3). That the HMM was relatively detergent resistant at the lower temperatures, but not after boiling, may indicate the existence of RcpA predominantly as an assembled multimer in the bacterium. The 37 and 65°C HMM-containing samples displayed only monomers upon boiling and electrophoresis (data not shown).

The same banding pattern of RcpA was observed with the derivatives of other clinical isolates of *A. actinomycetemcomitans*: DF2200N (serotype *a*), HK1651N (serotype *b*), and IDH781N (serotype *d*) (data not shown). In addition, we have found and sequenced a *tad* locus from *Aggregatibacter* (*Haemophilus*) *aphrophilus* (34, 39, 45; C. Sheth, C. Mott, A. Soczewska, P. J. Planet, and D. H. Figurski, unpublished data), and we were able to detect both the RcpA HMM and the monomer by probing *A. aphrophilus* cells with the antiserum specific for *A. actinomycetemcomitans* RcpA (data not shown). Together, these data provide the first biochemical evidence that the RcpA proteins in *tad* locus-containing organisms participate in a higher-ordered structure.

Localization of Rcp and Tad proteins to the bacterial membranes. The *rcpCAB* genes are predicted to code for proteins of 274, 460, and 167 residues, respectively, while the *tadCD* genes are likely to produce 288- and 253-amino-acid proteins (Fig. 1A). Predicted signal sequence cleavage sites of CU1000N RcpA (residue 29) and RcpB (residue 23) would produce proteins of approximately 46.9 and 16.4 kDa, respectively (18). Haase et al. (23) reported the migration of RcpA at 43 kDa and RcpB at 20 kDa in outer membrane fractions. We successfully generated polyclonal antisera to each of the three Rcp proteins and to the TadC protein. We also made two attempts to generate antiserum specific to TadD, but neither antiserum was able to detect the native protein in *A. actinomycetemcomitans* (data not shown).

The subcellular fractionation of *A. actinomycetemcomitans* was achieved by ultracentrifugation and selective solubilization of the inner membrane with the anionic detergent Sarkosyl. Previously, we used this method to demonstrate the localization pattern of the recombinant TadA-T7 protein (3). In addition, using this method with TadA-specific antiserum, we have since confirmed that native TadA localizes to the soluble and inner membrane fractions (data not shown).

Immunoblotting of subcellular fractions for RcpA, RcpB, and RcpC revealed that none of the Rcp proteins were present in detectable amounts in the buffer-soluble fraction, which consisted of cytoplasmic and, probably, periplasmic proteins (Fig. 4, lane S). RcpA and RcpC were observed in the inner membrane (Fig. 4, lane IM). All three Rcp proteins, including RcpB, were abundant in the Sarkosyl-insoluble outer membrane of *A. actinomycetemcomitans* (Fig. 4, lane OM). The TadC protein was not present in the soluble or outer membrane fractions but was contained exclusively in the inner membrane fraction (Fig. 4).

We used a plasmid construct, pJMM108, to determine the localization of TadD by expressing TadD-T7 in the *A. actinomycetemcomitans* CU1000N-derived *tadD* mutant. The open reading frames of *tadC* and *tadD* overlap, and previous plasmid constructs used to complement the *tadD* mutation included *tadC* for its Shine-Dalgarno sequence (29). In contrast, the pJMM108 plasmid (J. M. Morrison and D. H. Figurski, unpublished) provides a functional copy of *tadD* that initiates protein synthesis from a small region upstream of *tadC*, without the *tadC* open reading frame. This construct (*tadC* upstream region-*tadD*-T7 coding sequence) also includes a sequence encoding a T7 tag at the 3′ end of *tadD* so that the tagged protein (TadD-T7) can be visualized with anti-T7 epitope tag antiserum. The protein is functional because it complements the *tadD* mutation for the adherence-related phenotypes (J. M. Morrison and D. H. Figurski, unpublished).

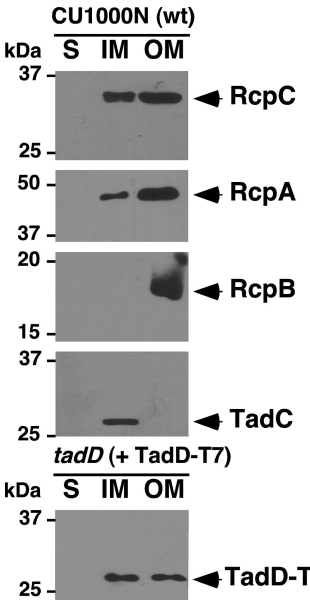

FIG. 4. Localization of Rcp proteins. Subcellular fractions of CU1000N (wild type [wt]) and Aa0727, the *tadD* mutant strain with added TadD-T7 (via pJMM108), were prepared by differential detergent solubilization of the inner membrane with 0.5% Sarkosyl, as described in the text. Immunoblots were probed with antiserum specific to RcpC, RcpA, RcpB, or TadC or to the T7 epitope tag. Arrows indicate the positions of RcpC, RcpA, RcpB, TadC, and TadD-T7. S, soluble fraction; IM, inner membrane; OM, outer membrane.

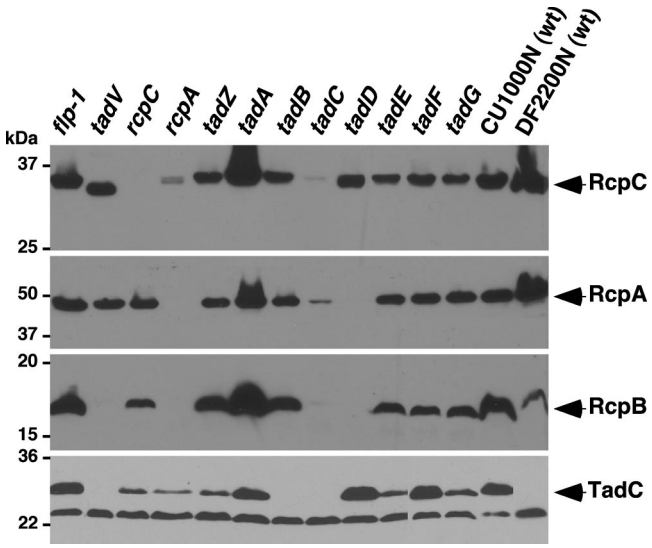

FIG. 5. Abundances of Rcp proteins in *tad* mutant strains. Whole-cell extracts of nonpolar (complementable) *tad* mutant strains and of wild-type (wt) strains CU1000N and DF2200N were prepared and analyzed by immunoblotting with anti-RcpC, anti-RcpA, anti-RcpB, or anti-TadC antiserum. The stronger signals from the *tadA* mutant strain were reproducible, despite equivalent protein concentrations (at A_{280}) in the extracts of the other strains. Arrows indicate the positions of RcpC, RcpA, RcpB, and TadC. In the TadC blot, the bottom band is nonspecific (see the text).

Immunoblots of the subcellular fractions prepared from the *tadD* strain complemented with TadD-T7 showed the presence of the tagged protein in the inner and outer membranes (Fig. 4). We were unable to detect TadD-T7 in the soluble fraction (Fig. 4), indicating that TadD does not remain in the cytoplasmic interior and is probably not in the periplasm either.

As expected from previous work (23), these results confirm the presence of RcpA and RcpB in the outer membrane. They also provide the first evidence for the localization of RcpC in both membranes and reveal that some RcpA protein may be observed in the inner membrane fraction. Additionally, TadC was localized to the inner membrane, and TadD-T7 was found in both of the bacterial membranes.

Other Tad proteins influence Rcp protein abundance. The association of *tad* locus proteins into a complex may be necessary for the operation of the Tad secretion system (45, 67). We reasoned that other proteins produced from the *tad* locus might influence the expression, abundance, and/or stability of the Rcp proteins. We compared the levels of production of the RcpA, RcpB, and RcpC proteins within nonpolar *tad* mutants (29, 31, 42, 45) to the levels produced by the parental, wild-type strains CU1000N and DF2200N. Our analysis did not include a mutant form of *flp-2*, as *flp-2* is not required for the production of Flp pili or for adherence, autoaggregation, and rough-colony formation by *A. actinomycetemcomitans* (42). We also did not include an *rcpB* mutant, since *rcpB* appears to be indispensable for the survival of otherwise *tad*$^+$ bacteria (42).

When immunoblots were probed with the RcpC-specific antiserum, RcpC was found as an abundant band in wild-type bacteria whereas no band was detected in the *rcpC* mutant, as expected (Fig. 5). We found that the abundance of the RcpC protein in the *rcpA* mutant was greatly reduced and that RcpC was nearly absent from the *tadC* mutant. The abundance of RcpC in other *tad* mutants was not significantly affected, although it was observed that the protein in the *tadV* strain migrated slightly faster than the RcpC proteins in the other strains (Fig. 5). Because the *tadV* strain was made in a DF2200N background (42), we compared its RcpC banding pattern to that of the DF2200N wild type. As the protein in the *tadV* strain, RcpC in the DF2200N wild type migrated faster than the RcpC proteins in the other strains analyzed. Therefore, the different migration is likely a property of the background strain.

The RcpA protein was absent in the *rcpA* mutant, and it was abundant in both wild-type strains (Fig. 5). In most other mutant strains, the intensity of the RcpA band was comparable to that of the band observed in wild-type *A. actinomycetemcomitans*. However, RcpA was visible only as a faint band in a *tadC* mutant and not at all in a *tadD* mutant (Fig. 5). When more-concentrated *tadD* samples were subjected to immunoblot analysis, RcpA could be detected, but only at very low levels (data not shown). This result indicates a severe defect in the production and/or stability of RcpA in the absence of TadD.

The anti-RcpB antiserum detected an abundance of native RcpB in wild-type CU1000N and in many of the CU1000N *tad* mutants, but RcpB was almost undetectable in the *rcpA*, *tadC*, and *tadD* mutant strains (Fig. 5). The anti-RcpB antiserum displayed poor recognition of the RcpB protein in both the DF2200N wild type and the *tadV* strain, which was made from DF2200N (Fig. 5). All of our antisera were generated using

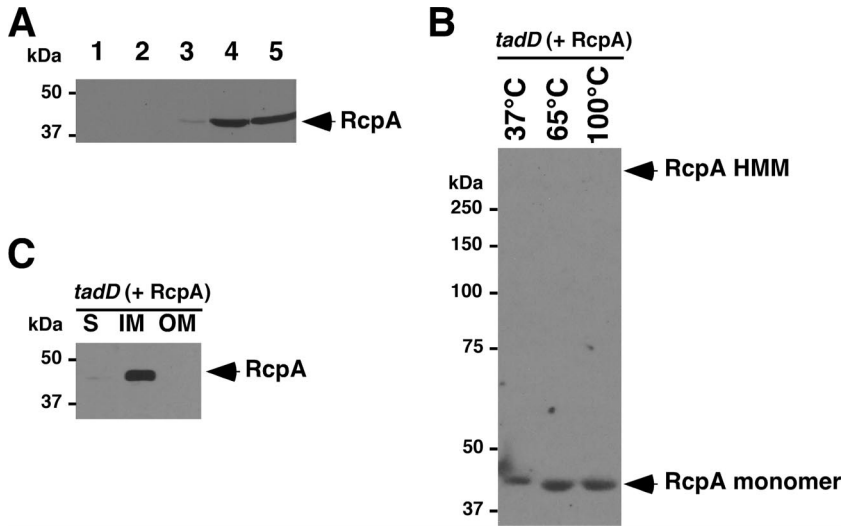

FIG. 6. TadD and its effects on RcpA. (A) Whole-cell extracts of the following strains were prepared and immunoblotted using anti-RcpA antiserum: lane 1, Aa1577 (*tadD* mutant); lane 2, Aa1758 (*tadD* mutant with empty vector); lane 3, Aa1759 (*tadD* mutant with RcpA); lane 4, Aa0727 (*tadD* mutant with TadD-T7); lane 5, CU1000N (wild type). (B) Whole-cell extracts of Aa1759 [*tadD* (+ RcpA)] were heated at 37, 65, or 100°C for 5 min in standard loading buffer and separated by SDS-PAGE through an 8% polyacrylamide gel. Immunoblotting was done with anti-RcpA antiserum. (C) Subcellular fractions of Aa1759 were prepared by differential detergent solubilization of the inner membrane with 0.5% Sarkosyl, and immunoblots were probed with anti-RcpA antiserum. S, soluble fraction; IM, inner membrane; and OM, outer membrane. Arrows indicate the positions of RcpA. In panel B, the RcpA HMM is indicated.

protein antigens from CU1000N. In contrast to RcpB, the RcpA and RcpC proteins were detected by their specific antisera in whole-cell extracts prepared from both CU1000N and DF2200N.

Because TadC appeared to be critical for the abundances of all three Rcp proteins, we tested whether or not the abundance of TadC was altered in the *tad* locus mutants, including *rcpA* and *rcpC*. Our TadC-specific antiserum recognized the native protein at the predicted size in CU1000N, as well as a lower, nonspecific band that was present in all of the strains we examined (Fig. 5). TadC was not detected in the *tadC* mutant, as expected. We found that in the absence of TadB, TadC abundance was severely diminished. TadC production and/or stability in several other mutants, including the *rcpA*, *rcpC*, *tadZ*, *tadE*, and *tadG* mutant strains, was less than that in the wild type (Fig. 5). The CU1000N TadC-specific antiserum failed to recognize its cognate protein in the DF2200N wild-type and *tadV* strains; therefore, as for RcpB, we were unable to determine the abundance of TadC in a *tadV* mutant.

In summary, we found defects in the production and/or stability of all three of the Rcp proteins in the *tadC* mutant and reciprocally diminished abundance of the TadC protein in *rcpC*- and *rcpA*-deficient strains. Additionally, RcpA and RcpB were nearly undetectable in the *tadD* mutant strain, and TadC was difficult to detect in the *tadB* mutant. RcpB and RcpC were detected at diminished levels in the *rcpA* mutant strain.

Importance of outer membrane protein TadD. The near absence of RcpA in the *tadD* mutant was dramatic. PSORT analysis (18) of the predicted CU1000N TadD protein sequence indicated that TadD may be a lipoprotein cleaved by signal peptidase II and localized to the outer membrane. Our results showed the localization of a tagged version of TadD in the outer membrane, though it was also detected in the inner membrane. Since some secretin proteins require an outer membrane lipoprotein for stabilization or membrane insertion (2), we considered that TadD may be an outer membrane docking protein for RcpA in the Tad system and that alterations in RcpA protein distribution may occur in its absence.

We did a complementation analysis to ascertain whether the production of TadD could restore the abundance of RcpA to the wild-type level. Immunoblots of whole-cell extracts showed that, whereas RcpA was severely diminished in the *tadD* mutant, the addition of TadD-T7 returned the abundance of RcpA to an intensity comparable to that in wild-type CU1000N (Fig. 6A). We also examined the production of RcpA in a *tadD* (plus RcpA) strain, which is the *tadD* mutant strain containing pPP40, a *tac*-mediated, RcpA-producing plasmid that complements an *rcpA* mutant (45). The *tadD* mutant strain contained only the chromosomal copy of *rcpA*, and although RcpA was detected in protein extracts of the *tadD* (plus RcpA) strain as a faint band (Fig. 6A), it seemed that additional copies of *rcpA* were not sufficient to restore wild-type levels of RcpA to the *tadD* mutant. We also observed that the addition of RcpA (vial pPP40) to the *tadD* mutant failed to restore adherence or rough colony morphology (data not shown).

We used the *tadD* (plus RcpA) strain to observe the oligomerization of RcpA in this mutant. Without TadD protein production, RcpA was no longer detected as an HMM but was present only in its monomeric form (Fig. 6B). In the *tadD* mutant, the RcpA protein was also no longer detected in the outer membrane but was present predominantly in the inner membrane fraction and was detected at low levels in the cytoplasm (Fig. 6C). RcpA did not exhibit this outer membrane defect in either the *tadC* or the *rcpC* mutant (data not shown), indicating that the mislocalization of RcpA in the *tadD* (plus RcpA) strain was because of the absence of TadD. Complementation of the *tadD* mutation restored both the localization pattern and the multimerization of RcpA (data not shown).

In summary, RcpA abundance could be fully restored by the complementation of the *tadD* mutation with TadD-T7. We did not detect RcpA at wild-type intensity without TadD production. In the absence of TadD, the outer membrane localization and oligomerization of RcpA were abolished.

DISCUSSION

The secretin family of outer membrane proteins form oligomeric channels through which fully folded and assembled secretion substrates exit the cell. RcpA has been assumed to operate as the secretin for the Flp pilus secretion system because of (i) the similarity of the sequence of its C terminus to those of members of the secretin superfamily, (ii) the detection of this protein in *A. actinomycetemcomitans* outer membranes (23), and (iii) the genetic requirement for a functional *rcpA* gene for Flp pilus production (45). Here, we have shown that the RcpA protein of *A. actinomycetemcomitans* exhibits an evolutionary relationship with known secretins, and we have shown patterns of membrane localization and multimerization typical of well-studied secretins.

Our parsimony-based phylogenetic analysis of 186 genes, which included measurements of nodal support, is the largest secretin gene superfamily phylogeny to date (69). We found that the *rcpA* gene and its close homologs form a distinct, major monophyletic subfamily with an ancient divergence from other well-known secretin genes. The *rcpA* subfamily groups with other secretin genes from T2S systems, which is consistent with the functional similarities reported here and with the numerous similarities between T2S systems and the Tad secretion system (67). Indeed, we have proposed that the Tad secretion system be classified as a T2S system (44, 67). The ancient divergence of the *rcpA* family indicates that these functional similarities are very strongly selected and conserved.

Association with *tad* locus genes and participation in Flp pilus assembly are the exclusive properties of *rcpA* subfamily genes. In all cases, genes of the *rcpA* subfamily are found in close proximity to at least some other *tad* locus genes, and such associations are not seen outside the *rcpA* clade, indicating that the *rcpA* subfamily is representative of a historical event in which the ancestral *rcpA* secretin gene became associated with the *tad* locus. The sister group of the *rcpA* subfamily, which is composed of chromosomal and plasmid-borne secretin genes from *R. solanacearum*, is associated with secretion genes only distantly related to *tad* locus genes. This observation indicates that the initial event that created the *rcpA* lineage may have been the addition of a secretin gene from another secretion system to a preexisting *tad* locus. It is possible that this was a plasmid-mediated event because several of the closest relatives in *R. solanacearum* are found on megaplasmids.

The secretin N terminus is proposed to reside at least partially in the periplasm, where it may interact with other secretory proteins or physically gate the opening of its own channel (13, 57). In our alignment (Fig. 1B), the N terminus of RcpA was noticeably shorter than those of the proteins CpaC, its close homolog, and PulD. The relative shortness of the N terminus of RcpA may argue against a self-gating function, and another protein in the Tad system may be required. Additionally, our observation that the overexpression of *rcpA* is toxic in both *A. actinomycetemcomitans* and *E. coli* (data not shown) may be interpreted as an inability of RcpA to provide a gate on its own, resulting in detrimental effects such as membrane leakiness.

Mounting evidence leads us to suggest that RcpB may be required in the Tad secretion system as a gating protein. (i) The necessity for *rcpB* in *tad*$^+$ bacteria implies that something about the composition of an *rcpB*-deficient Tad system causes toxicity to the point of lethality. (ii) RcpB is in the outer membrane and has been observed in cross-linked homomultimers (data not shown), much like RcpA. (iii) RcpB abundance is dependent on the presence of the RcpA secretin, and like the secretin, RcpB was less abundant and no longer localized to the outer membrane in the *tadD* mutant (data not shown). Finally, (iv) our phylogenetic analysis offers support for the requirement of an interacting protein encoded by a gene with sequence similarity to *rcpB* or to *cpaD*, which immediately follows every *rcpA* subfamily gene we examined.

Protein abundance patterns have been used successfully to deduce protein interactions within transport systems (4, 27, 50, 51, 58). It is possible that the loss of stabilizing physical interactions in *tad* mutant strains may account for some of the abundance defects we observed. Consequently, an abundance defect for a noncognate protein in a *tad* mutant may represent the degradation of that protein in the absence of interaction with the missing gene product. Reciprocal abundance relationships have been observed for TadC-RcpC and TadC-RcpA, predicting that there may be stabilizing or complex-related contacts occurring. In addition, all three Rcp proteins were less abundant in a *tadC* mutant than in the wild type, RcpA and RcpB were nearly absent in a *tadD* mutant, and RcpB and RcpC seemed to depend on the presence of RcpA.

We found TadC to be a 32-kDa inner membrane protein. In homology searches, both *A. actinomycetemcomitans* TadB and TadC show similarity to PilC-like proteins, which are integral inner membrane members of T2S systems (41). TadB and TadC may associate to form a heteromultimer that allows the passage of other Tad components across the inner membrane (8). In support of a possible association, we found that TadC protein levels in the *tadB* mutant were severely depleted, while many other mutants exhibited wild-type levels. We have not been able to examine the expression profile or localization of TadB because we were not able to make an antiserum that recognizes the native protein in *A. actinomycetemcomitans*, and attempts to detect a T7-tagged fusion were not successful.

The RcpC/CpaB homologs, including those in *H. ducreyi*, *P. multocida*, *C. crescentus* (CpaB), and *Pseudomonas* spp. (CpaB) contain two β-clip motifs (28). While β-clip folds were previously known to function in fish antifreeze proteins, in sialic acid synthase, and in a range of other enzymes, it has been proposed that in RcpC/CpaB proteins they may function by binding carbohydrate moieties of peptidoglycan to assemble structures like Flp pili or flagella. Our finding (66) that the Flp1 protein migrates faster in an *rcpC* mutant than in the wild-type strain or in the other *tad* mutants indicates that the RcpC protein may be involved in the known glycosylation of Flp1, and this study has revealed that the RcpC protein may function in association with one or both bacterial membranes.

Our immunoblots showed RcpC, RcpA, and TadD-T7 in both the outer membrane fraction and the Sarkosyl-soluble inner membrane. The detection of these proteins in the inner

membrane may be a manifestation of their trafficking to the outer membrane or of their extraction resulting from interactions at the outer face of the inner membrane, as has been suggested previously for other systems (50, 55). Contamination of fractions is not a factor (unpublished results). Evidence of outer membrane proteins in the cytoplasmic membrane was also found in localization studies of the secretins PulD and TcpC (4, 9), and evidence of intermembrane interactions for many secretin proteins has been supported biochemically (11, 27, 46, 47, 51).

Outer membrane lipoproteins have been implicated in the assembly of secretin oligomers, in outer membrane insertion of secretins, and in the general stabilization of assembled secretin complexes. Monomeric RcpA in the *tadD* mutant was difficult to detect, even when extra copies of *rcpA* were provided in *trans*. One possibility is that without TadD there is severe destabilization and probably near-complete degradation of the RcpA protein. Although we cannot yet separate the effects, our multimerization and localization results indicate that TadD may mediate the association of RcpA subunits and act as an assembly factor for the insertion of RcpA into the outer membrane. The docking proteins PilW and Tgl, both lipoproteins, do this for their secretins (6, 40). They each contain TPR sequences that are thought to be important for interactions during secretin complex assembly (40). The TadD sequence contains a 34-residue TPR domain that may mediate an interaction with the RcpA protein.

Through localization, protein stability, and additional analyses, this study has started to uncover potential functions of membrane-bound Tad proteins in Flp pilus biogenesis and likely relationships to other proteins within the secreton. Since its component proteins seem to be interdependent for stability, the Tad secretion system probably forms a complex that spans both membranes, and this complex may include an outer membrane module. It is clear that the continued study of these proteins will help to define the mechanism by which the Tad secreton operates.

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