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The *Pseudomonas aeruginosa* Secreted Protein PA2934 Decreases Apical Membrane Expression of the Cystic Fibrosis Transmembrane Conductance Regulator[▽]

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We previously reported that *Pseudomonas aeruginosa* PA14 secretes a protein that can reduce the apical membrane expression of the *c*ystic *f*ibrosis *t*ransmembrane conductance *r*egulator (CFTR) protein. Here we report that we have used a proteomic approach to identify this secreted protein as PA2394, and we have named the gene *cif*, for *C*FTR *i*nhibitory *f*actor. We demonstrate that Cif is a secreted protein and is found associated with outer membrane-derived vesicles. Expression of Cif in *Escherichia coli* and purification of the C-terminal six-His-tagged Cif protein showed that Cif is necessary and sufficient to mediate the reduction in apical membrane expression of CFTR and a concomitant reduction in CFTR-mediated Cl^- ion secretion. Cif demonstrates epoxide hydrolase activity in vitro and requires a highly conserved histidine residue identified in α/β hydrolase family enzymes to catalyze this reaction. Mutating this histidine residue also abolishes the ability of Cif to reduce apical membrane CFTR expression. Finally, we demonstrate that the *cif* gene is expressed in the cystic fibrosis (CF) lung and that nonmucoid isolates of *P. aeruginosa* show greater expression of the gene than do mucoid isolates. We propose a model in which the Cif-mediated decrease in apical membrane expression of CFTR by environmental isolates of *P. aeruginosa* facilitates the colonization of the CF lung by this microbe.

The bacterium *Pseudomonas aeruginosa*, a known pathogen of a variety of organisms, has the ability to infect individuals who are immunocompromised due to injury or disease (19, 34, 52). Of prime interest to those studying *P. aeruginosa* is its role in the human genetic disease cystic fibrosis (CF). Up to 90% of individuals suffering from CF become infected with *P. aeruginosa* during their lifetime, and this organism is the leading cause of morbidity and mortality among CF patients (5, 17). In the majority of cases, colonization of the CF airway by *P. aeruginosa* leads to a chronic infection that is refractory to antimicrobial therapy (8, 22).

The disease CF is the result of mutations within the *c*ystic *f*ibrosis *t*ransmembrane conductance *r*egulator (CFTR), the most common of which is a deletion of the phenylalanine at residue 508 (ΔF508) (18, 41, 42, 50). CFTR localizes to the apical membrane of epithelial tissues, where it directly regulates the flux of chloride ions across the apical membrane and indirectly regulates the flux of sodium ions and water (25, 41). The ΔF508 mutation results in mislocalization of CFTR from the apical membrane and thus alters the flux of ions and water across this membrane. Individuals homozygous for this allele demonstrate altered sodium and chloride secretion, leading to an increase in mucus and air-surface liquid viscosity and a general decrease in air-surface liquid height. This increased viscosity as well as the ensuing ciliastasis allow for the colonization of the lung by a variety of microbes (5). These initial infections eventually cede to *P. aeruginosa*, which establishes the aforementioned chronic infection.

The ability of *P. aeruginosa* to colonize the CF airway and rapidly become the dominant organism has been studied thoroughly and yet is still poorly understood. It is likely that the infection process is multifactorial. For example, the secretion of well-characterized virulence factors, such as elastase and pyocyanin, results in tissue damage and eradication of other microbes (6, 27, 29, 53), perhaps allowing *P. aeruginosa* to dominate the CF airway. Alternatively, it has been proposed that *P. aeruginosa* directly interacts with CFTR (39). A recent model proposes that *P. aeruginosa* initially colonizes the lung as a free-swimming bacterium but quickly begins to form complex communities embedded in an exopolymeric matrix, known as biofilms, which demonstrate significantly more resistance to antimicrobial chemotherapy than do their planktonic counterparts (16, 21, 46).

We have evidence that *P. aeruginosa* may exacerbate the problems associated with decreased CFTR function. Previous work by our group demonstrated that *P. aeruginosa* secretes a factor capable of reducing apical membrane expression of both wild-type (WT) CFTR and ΔF508-CFTR, termed Cif (*C*FTR *i*nhibitory *f*actor) (48). We present here the purification, identification, and characterization of Cif, a protein encoded by the PA2934 locus of *P. aeruginosa* PA14.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. All of the bacterial strains and plasmids used in this study are shown in Table 1. All bacterial strains were grown in lysogeny broth (LB) (3) unless otherwise noted. The growth medium was supplemented with antibiotics at the following concentrations: gentamicin, 10 μg/ml (*Escherichia coli*) or 100 μg/ml (*P. aeruginosa*); and ampicillin, 150 μg/ml (*E. coli*)

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TABLE 1. Strains, plasmids, and primers used for this study

Strain, plasmid, or primer	Relevant genotype, description, or sequence (5′–3′)	Source or reference
Strains		
P. aeruginosa strains		
PA14	Wild type	40
SMC3498	PA14 PA2934 single-crossover mutant	This study
SMC3499	PA14 PA4476 single-crossover mutant	This study
SMC3500	PA14 PA1914 single-crossover mutant	This study
SMC3501	PA14 plus pMQ70	This study
SMC3502	PA14 ΔPA2934	This study
SMC3503	SMC3502 plus pDPM73 (PA2934-His)	This study
SMC3504	SMC3502 plus pMQ70	This study
SMC3505	*E. coli* Top10 plus pDPM73 (PA2934-His)	This study
SMC3506	SMC3498 plus pDPM73 (PA2934-His)	This study
SMC3507	*E. coli* Top10 plus pDPM77 (PA2934-His,H269A)	This study
SMC3510	*E. coli* S17 plus pDPM74	This study
SMC1584	Clinical isolate, nonmucoid	This study
SMC1585	Clinical isolate, mucoid	This study
SMC1586	Clinical isolate, nonmucoid	This study
SMC1587	Clinical isolate, mucoid	This study
SMC1588	Clinical isolate, nonmucoid	This study
SMC1589	Clinical isolate, mucoid	This study
SMC1590	Clinical isolate, mucoid	This study
SMC1591	Clinical isolate, nonmucoid	This study
SMC1593	Clinical isolate, nonmucoid	This study
SMC1596	Clinical isolate, mucoid	This study
E. coli strain		
Top10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) f80*lacZ*Δ*M15* Δ*lacX74 recA1 ara*Δ*139* Δ(*araleu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Plasmids		
pDPM60	PA2934 fragment in pMQ89; Gm^r	This study
pDPM61	PA4476 fragment in pMQ89; Gm^r	This study
pDPM66	PA1914 fragment in pMQ89; Gm^r	This study
pDPM70	WT PA2934 in pMQ71; Cb^r Ap^r	This study
pDPM73	PA2934 histidine fusion in pMQ70; Cb^r Ap^r	This study
pDPM77	Derivative of pDPM73 carrying H269A mutation	This study
pMQ89	Single-crossover mutation vector; Gm^r	45
pMQ30	*P. aeruginosa* suicide vector for clean deletions; Gm^r	45
pMQ89	Single-crossover mutation vector; Gm^r	45
pMQ70	Arabinose-inducible expression vector; Cb^r Ap^r *URA3*	45
Primers		
PA2934_SXO_for	GAGGAATTCCCCGTCCCGAATGGCTTCG	
PA2934_SXO_rev	CCGCGGATCCGCCAGACCAGCGACTCGCCCTGGGCG	
PA4476_SXO_for	CCGCGGATCCGTCCTGGGTCTGTGCGCCCTGGTACTGG	
PA4476_SXO_rev	CCGCGAATTCCGCGCCGACCGCGCTGAGCGACAGCG	
PA1914_SXO_for	CGCCGGATCCGGCCTGGCGCTGACACCCGCGGCCC	
PA1914_SXO_rev	CCGCGAATTCGCACTGCCGCGAATGGTCGCCGCGG	
PA2934_KO_1	GGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCCGCAAGCGG CTGTTCGTCGCCTGCC	
PA2934_KO_2	CTGATCGCCCGGACCGGGACGACGGTTGTGCTTCCTTGGTGGGGTC	
PA2934_KO_3	GACCCCACCAAGGAAGCACAACCGTCGTCCCGGTCCGGGCGATCAG	
PA2934_KO_4	GTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCCGAACAGCGCG GTGTCCTTGAAGC	
PA2934_comp_for	CCGCGCTAGCCCAAGGAAGCACAACCATGATCC	
PA2934_comp_rev	CCGCGGTACCTCAGCGACCGCGGCTGAGGAAG	
PDPM70-His	GTATCAGGCTGAAAATCTTCTCTCATCCGCCTCAATGATGATGATGATGATG GCGACCGCGGCTGAGGAAGTCGATCACCAGG	
PA2934_mut_out_for	GATTTAATCTGTATCAGGCTGAAAATCTTCTCTCATCCGCCTCAATGATGATG ATGATGATGGCG	
H269A_rev	CCATGACCCTCGCCGGCGGCGGCGCCGGCGGCATGGGCACGTTCCAGCTGG	
H269A_for	CCAGCTGGAACGTGCCCATGCCGCCGGCGCCGCCGCCGGCGAGGGTCATGG	
PA2934_mut_out_rev	CCATGACCCTCGCCGGCGGCGGCGCCGGCGGCATGGGCACGTTCCAGCTGG	
PA2934_rt_1	CTCCTGGCCGGCATCGCCCTGACCTTCTCC	
PA2934_rt_2	CCATTCGTACCAGGTCTGGCCGAAGCCGTGC	
RplU_rt_1	GGTGGCAAGCAGCACAAAGTCACCG	
RplU_rt_2	GCGGACCTTGTCGTGACGGCCGTGG	

or 1.5 mg/ml (*P. aeruginosa*). All strains were grown at 37°C. Yeast cultures were grown in either rich (yeast extract-peptone-dextrose) or minimal (SD−Ura; Sunrise Science Products, San Diego, CA) medium at 30°C. All restriction enzymes were purchased from New England Biolabs (Beverly, MA). All plasmids were constructed in *E. coli* Top10, using standard protocols, or *Saccharomyces cerevisiae* InvSc (Invitrogen, Carlsbad, CA), using in vivo recombination, and electroporated or conjugated into *P. aeruginosa* strain PA14 as reported previously (10, 45).

Purification of Cif. Five-milliliter cultures of *P. aeruginosa* PA14 were grown overnight at 37°C, followed by 1:1,000 dilution into 100 ml LB. Cultures were grown with shaking at 37°C for 18 h. Supernatants were harvested by centrifugation at 7,000 × *g* for 15 min followed by filtration through a 0.22-μm filter. Sterile supernatants were concentrated 10-fold using Amicon Centriprep centrifugation filters per the manufacturer's instructions (Millipore, Billerica, MA). Concentrated supernatants were dialyzed against 4 liters of 25 mM morpholineethanesulfonic acid buffer, pH 6.5, for 2 h, using Pierce Slide-a-lyzers with a 10-kDa cutoff (Pierce, Rockford, IL). Samples were then fractionated utilizing a 1-ml Amersham Biosciences HiTrap Q FF anion-exchange chromatography column (Amersham Biosciences, Uppsala, Sweden), using a stepwise gradient of 0 and 50 mM and 2 M NaCl. Collected fractions were then dialyzed against 4 liters of phosphate-buffered saline and utilized in the apical CFTR membrane expression assays described below.

MudPIT analysis of active fractions. Samples for multidimensional protein identification technology (MudPIT) analysis were lyophilized using a Savant SC110 Speed-Vac and stored on ice. Samples were submitted to the Keck Proteomics and Mass Spectrometry facility at Yale University for MudPIT analysis. The resulting data were analyzed utilizing Mascot and Seaquest software, and ion masses were compared to those in the available *P. aeruginosa* PA14 protein database (NCBI accession no. nr 20040730). MudPIT separates proteins using columns consisting of strong cation-exchange resin in series with reverse-phase resin. Through cycles of increasing salt and hydrophobicity, peptides are eluted from the tandem column, resulting in high resolution of the peptides. The mass spectrometer isolates peptides as they elute and subjects them to collision-induced dissociation, recording the fragment ions in a tandem mass spectrum. These spectra are matched to the aforementioned database peptide sequences by using the SEQUEST algorithm.

Cell lines and cell culture. Madin-Darby canine kidney (MDCK) cells stably expressing a green fluorescent protein–WT CFTR fusion protein (WT-MDCK cells) were established and maintained in culture in a 5% CO_2-95% air incubator at 37°C as described previously (37, 38). The addition of green fluorescent protein to the N terminus of CFTR had no effect on CFTR localization, trafficking, function as a Cl^- channel, or degradation (37). WT-MDCK cells were seeded onto Transwell permeable supports (0.2×10^6 cells on 24-mm-diameter supports with a 0.4-μm pore size) and grown in culture at 33°C for 7 days as polarized monolayers. Sodium butyrate (5 mM; Sigma-Aldrich) was added to the WT-MDCK cell culture medium 16 to 18 h before experiments to stimulate CFTR expression.

Parental human bronchial epithelial CFBE41o$^-$ cells (ΔF508/ΔF508), originally immortalized and characterized by Gruenert and colleagues (7, 11), were stably transduced with WT CFTR (WT-CFBE cells; a generous gift from J. P. Clancy, Department of Pediatrics, University of Alabama at Birmingham, Birmingham) (2). WT-CFBE cells were cultured in a 5% CO_2-95% air incubator at 37°C as previously described (48). To establish polarized monolayers, 1×10^6 CFBE41o$^-$ cells were seeded onto 12-mm Snapwell or 24-mm Transwell permeable supports (0.4-μm pore size; Corning) and grown in air-liquid interface culture at 37°C for 7 to 9 days.

Determination of CFTR expression in the apical plasma membrane. To identify active fractions during Cif purification, as well as to assay the Cif activity of various strains and the purified protein, we used a previously described surface biotinylation assay (48). Unless otherwise noted, 50 μg of purified PA2934-His was used in all surface biotinylation experiments.

Ussing chamber measurements. Monolayers grown on 12-mm-diameter Snapwell permeable supports as described above were mounted in an Ussing-type chamber (Physiological Instruments, San Diego, CA) and bathed in solutions that were maintained at 37°C and pH 7.4 and stirred using bubbling with 5% CO_2-95% air. The apical bath solution contained 5 mM NaCl, 115 mM sodium gluconate, 25 mM $NaHCO_3$, 3.3 mM KH_2PO_4, 0.8 mM K_2HPO_4, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 10 mM mannitol. The basolateral bath solution contained 120 mM NaCl, 25 mM $NaHCO_3$, 3.3 mM KH_2PO_4, 0.8 mM K_2HPO_4, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 10 mM glucose. Short-circuit current (I_{sc}) was measured by clamping the transepithelial voltage across the monolayers to 0 mV by using a voltage clamp (model VCC MC6; Physiological Instruments) as described previously (20, 31, 48). Current output from the clamp was digitized using an analog-to-digital converter (iWorx, Dover, NH). Data collection and analysis were performed using LabScribe, version 1.8, software (iWorx).

To determine the effects of purified, recombinant PA2934 on the CFTR-mediated Cl^- current (I_{sc}), forskolin (20 μM) was added to the apical and basolateral solutions to stimulate I_{sc}, and then 50 μg of purified PA2934-His in buffer or buffer alone (20 mM HEPES buffer, pH 7.5, containing 500 mM NaCl) was added to the apical bath solution. An equal volume of buffer was also added to the basolateral side to maintain the apical-to-basolateral Cl^- concentration gradient. $CFTR_{inh}$-172 (5 μM), a known inhibitor of CFTR activity (32), was added to the apical solution as a positive control for inhibition of CFTR Cl channels. I_{sc} data are expressed as changes in forskolin-stimulated I_{sc}.

Molecular techniques. All molecular techniques were performed as previously described (47). Single-crossover mutations of the PA1914, PA2934, and PA4476 loci were performed utilizing suicide plasmids based on pMQ89 (45). The deletion of the PA2934 gene was done using previously described methods (30). Plasmids were propagated in *E. coli* S17 and conjugated into *P. aeruginosa* strain PA14 as previously described (9). Recombinant strains were selected for and maintained in medium supplemented with gentamicin.

Plasmid construction. Plasmids pDPM60, pDPM61, and pDPM66 contain ~400-bp internal regions of the PA2934, PA4476, and PA1914 genes, respectively. The following primers were used to amplify the internal regions of the PA2934, PA4476, and PA1914 genes: PA2934_SXO_for, PA2934_SXO_rev, PA4476_SXO_for, PA4476_SXO_rev, PA1914_SXO_for, and PA1914_SXO_rev. Primers were engineered to create an amplicon that could be digested with EcoRI and HindIII and ligated into the suicide vector pMQ89 digested in a similar manner.

Construction of the PA2934 deletion plasmid was performed as previously described (30), using primers PA2934_KO_1, PA2934_KO_2, PA2934_KO_3, and PA2934_KO_4. Amplicons were created using primer pairs KO_1-KO_2 and KO_3-KO_4 and were recombined using *S. cerevisiae* InvSc1 (Invitrogen) into plasmid pMQ30 linearized with restriction enzymes EcoRI, HindIII, and BamHI.

Plasmid pDPM70, containing the full-length WT PA2934 open reading frame, was constructed by amplifying the PA2934 gene with primers PA2934_comp_for and PA2934_comp_rev, followed by digestion with EcoRI and NheI. The digested amplicon was ligated into pMQ70 (45), which was previously digested in a similar manner. pDPM73 was created utilizing in vivo recombination cloning as previously described (45), using pDPM70 digested with NheI and the primer PDPM70-His, resulting in an in-frame, C-terminal hexahistidine tag fusion to the PA2934 protein. Plasmid pDPM77, which expresses the PA2934-His protein with an H269A mutation, was constructed using a similar technique. The PA2934 open reading frame was amplified from pDPM73, using primer pairs PA2934_mut_out_for-H269A_rev and H269A_for-PA2934_mut_out_rev. The resulting amplicons were utilized in the in vivo recombination cloning technique, utilizing linearized pMQ70 as the plasmid backbone.

Purification of PA2934-His protein. *E. coli* Top10 strain SMC3505, expressing a C-terminal hexahistidine variant of PA2934 (PA2934-His) from plasmid pDPM73, was grown overnight at 37°C under selection. Cultures were diluted 1:100 in 4 liters of LB medium supplemented with ampicillin and 0.2% arabinose and grown for 8 h at 37°C with shaking. Following growth, culture supernatants were harvested by centrifugation of bacteria at 7,000 × *g* for 20 min. Supernatants were filtered through a 0.22-μm filter to remove any bacteria remaining in suspension. Sterile supernatant was then fractionated utilizing an Amersham HisTrap FF 5 ml nickel-affinity column. Protein was eluted over a 20 to 500 mM imidazole gradient, with the bulk of the purified PA2934-His protein eluting at ~100 mM. Fractions containing the protein were concentrated using an Amicon Ultra 15 centrifugation filter with a cutoff of 30 kDa per the manufacturer's instructions, followed by dialysis against 20 mM HEPES buffer, pH 7.5, containing 500 mM NaCl. The protein concentration was determined utilizing a Bio-Rad protein assay kit (Hercules, CA). Purity was determined by resolving the sample by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detecting it by Coomassie blue staining.

Epoxide hydrolase activity assay. Purified PA2934-His was assayed for the ability to degrade the epoxide hydrolase synthetic substrate (2*S*,3*S*)-*trans*-3-phenyl-2-oxiranylmethyl 4-nitrophenyl carbonate [(*S*)-NEPC; Sigma, St. Louis, MO]. PA2934-His was incubated in 20 mM Tris, pH 8.4, 500 mM NaCl with 100 μM (*S*)-NEPC in a final volume of 200 μl at 37°C for 60 min. Degradation of (*S*)-NEPC was measured as an increase in absorbance (optical density at 405 nm [OD_{405}]), utilizing a Molecular Devices SpectraMax M2 plate reader. Reactions were normalized to controls lacking enzyme, accounting for spontaneous hydrolysis of the substrate.

RNA purification from bacterial cells and cDNA synthesis. Strains for quantitative real-time reverse transcription-PCR (qRT-PCR) were grown overnight in LB medium and subsequently diluted 1:100 in LB medium and grown to an

OD_{600} of 2.5. Five-hundred-microliter aliquots of cultures were harvested and centrifuged at 16,000 × *g* for 2 minutes, and the cell pellets were frozen at −80°C. Strains were grown in triplicate, and two samples were harvested per replicate. RNAs were isolated using a QIAGEN RNeasy kit per the manufacturer's instructions. Following initial purification, samples were incubated with RNase-free DNase for 1 h at 37°C to further remove contaminating DNA and were repurified using a QIAGEN RNeasy kit. DNA contamination was assessed by using samples in a standard PCR assay. The RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer (Wilmington, DE). cDNAs were amplified utilizing a Superscript III first-strand polymerase kit (Invitrogen, Carlsbad, CA) per the manufacturer's instructions, utilizing random hexamers to prime the reactions. One microgram of RNA was used for cDNA synthesis.

RNA purification from sputum and cDNA synthesis. Respiratory sputa were collected from two individuals with CF and divided such that one sample was subjected to clinical microbiological analysis while the other was used for the purposes of RNA isolation. Both sputum samples contained *P. aeruginosa*, based on microbiological culture analysis in the DHMC clinical laboratory. After collection, an equal volume of RNALater, an RNA stabilizing agent, was added to each sample prior to storage at −80°C. To isolate total sputum RNA, samples were thawed on ice and centrifuged for 10 min at 13,000 rpm. The pellet was resuspended, followed by homogenization by passage through a 24-gauge syringe needle several times. Subsequent RNA purification steps were performed with a QIAGEN RNeasy kit according to the manufacturer's protocols. Contaminating DNA was removed using an Ambion DnaseFree kit according to the manufacturer's instructions. RNAs were analyzed spectrophotometrically to determine their concentration, and the absence of DNA was assessed by real-time RT-PCR analysis. For cDNA synthesis, 500 ng of RNA was used as a template in a cDNA synthesis reaction with Superscript III, using random primers [$(NS)_5$] according to the manufacturer's instructions; no-reverse-transcriptase controls were included in all analyses.

qRT-PCR. qRT-PCRs were carried out as previously described (30). Primers were engineered to amplify the first 100 base pairs of the PA2934 open reading frame. The *rplU* gene served as a control for all qRT-PCRs, as previous reports have demonstrated that its transcription is independent of growth stage or conditions across all *P. aeruginosa* strains assayed to date (30, 33). The data are expressed as either relative input cDNA levels (in picograms) of *cif* transcript compared to *rplU* transcript or total quantities of cDNA input for either the *cif* or *rplU* gene. Input cDNA levels were determined by comparing cycle threshold (C_T) values obtained during the reactions to those obtained from a standard DNA curve specific for either the *cif* or *rplU* gene. All qRT-PCRs were performed using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA).

Vesicle fractionation. Vesicles were purified from an overnight culture of *P. aeruginosa* PA14 grown to stationary phase by using one of the following methods. The first method was modified from the procedure reported by Bauman and Kuehn (1). Cells were removed by centrifugation, and supernatants were filtered through a 0.45-μm filter, followed by centrifugation at 39,000 × *g* for 1 h at 4°C. Pellets from centrifugation were resuspended in 50 mM HEPES buffer, pH 6.8, and adjusted to 45% Optiprep in 10 mM HEPES containing 0.85% NaCl, pH 7.4 (wt/vol). Discontinuous Optiprep gradients were then layered over the vesicle samples as follows: 0.8 ml 40% Optiprep, 0.8 ml 35% Optiprep, 1.6 ml 30% Optiprep, and 0.8 ml 20% Optiprep in 10 mM HEPES–0.85% NaCl buffer. Gradients were centrifuged at 100,000 × *g* for 18 h at 4°C. Immediately following centrifugation, 500-μl fractions were collected from the gradients. Proteins in each fraction were precipitated with 10% trichloroacetic acid (TCA) and visualized by 12% SDS-PAGE and Coomassie blue staining.

Alternatively, we utilized a modified version of protocols published by Horstman and Kuehn (24) and Demuth et al. (12). Cells were removed by centrifugation, and supernatants were filtered through a 0.45-μm filter, followed by concentrating the sample with an Amicon Ultra 15 30-kDa-cutoff membrane filter by centrifugation at 4,000 × *g* for 15 min. The filtered supernatant was centrifuged at 45,000 × *g* for 1 h; the pellet was resuspended in 4 ml of 20 mM HEPES, pH 7.4, plus 500 mM NaCl, followed by a second centrifugation at 45,000 × *g* for 1 h; and the pellet was again resuspended in 4 ml of 20 mM HEPES, pH 7.4, plus 500 mM NaCl. Twenty microliters of 1.5% deoxycholate and 200 μl 72% TCA were added to 1 ml of the vesicle fraction and incubated on ice for 10 min, followed by centrifugation at 16,000 × *g* for 15 min at room temperature. The pellet was washed twice with ice-cold 70% ethanol, and the residual ethanol was evaporated by incubation at 65°C. The pellet was resuspended in 200 μl of 1× SDS running buffer, and a 10-μl sample was resolved by SDS-PAGE and Western blotting, performed as reported previously (9). Similar results were obtained using the two techniques.

Antibodies. Polyclonal rabbit anti-Cif antibodies were produced by Covance Research Products (Denver, PA). Briefly, hexahistidine-tagged Cif was purified as described above, using nickel-affinity chromatography, and diluted to a final concentration of 1 mg/ml. The purified protein was then provided to Covance Research Products for immunizations. Sera from day 51 bleeds were used at 1:1,000, using standard Western blotting techniques (9).

Antibody to the outer membrane protein OprF was produced against a partially purified outer membrane fraction from *P. aeruginosa*. Specific cross-reactivity to the OprF protein was determined in two ways. First, vesicles were prepared by using the Optiprep gradient described above. The gradient fraction shown previously to contain the suspected OprF cross-reacting band was TCA precipitated, the proteins were resolved by SDS-PAGE, and a gel slice corresponding to ~37 kDa was excised and analyzed by mass spectroscopy at the Dartmouth Proteomics Shared Resource. Five peptides matching OprF were identified in this sample. Second, the loss of a cross-reacting band of the appropriate size (~37 kDa) was observed for an *oprF* mutant strain. Nobuhiko Nomura, Graduate School of Life and Environmental Sciences, University of Tsukuba, generously provided the polyclonal antibody prior to publication.

RESULTS

Purification of Cif activity. Previous work by our group demonstrated that exposing epithelial cells to *P. aeruginosa* strain PA14 reduces apical membrane expression of CFTR (48). We named the factor(s) responsible for this activity Cif (48). Our published studies show that cell-free culture supernatants also reduce apical membrane CFTR expression, thus suggesting that Cif is secreted.

To determine the identity of Cif, we fractionated cell-free supernatants from stationary-phase cultures, utilizing anion-exchange chromatography and a step gradient of NaCl, and the resulting fractions were assayed for the ability to decrease apical membrane expression of CFTR in WT-CFBE cells. A crude supernatant of *P. aeruginosa* PA14 reduced apical membrane CFTR expression to ~20% of the WT level in this experiment (Fig. 1A). Upon fractionation of the supernatant, we found that the two flowthrough fractions (F1 and F2) and the fraction resulting from washing the column with 0 mM NaCl (wash) lacked Cif activity. However, the Cif activity was eluted from the column with 50 mM NaCl. Fraction E1 resulted in <5% of WT apical membrane CFTR expression (Fig. 1A, fraction E1). E2, a subsequent second fraction eluted with 2 M NaCl, also showed some Cif activity. The marked increase in Cif activity observed in the E1 fraction compared to that in the unfractionated supernatant was likely due to an increased concentration of the factor in this fraction compared to that in the crude supernatant, resulting in an increase in Cif activity.

SDS-PAGE analysis of the fractions demonstrated that while fraction E1 was less complex than the crude supernatant, it still contained approximately 25 proteins (Fig. 1B).

Identification of PA2934 as Cif. We next identified the proteins in the Cif-containing fractions by utilizing MudPIT. Peptide masses from the MudPIT analysis were compared to those in the *P. aeruginosa* PA14 protein database (NCBI accession no. nr 40070473), resulting in the identification of 20 proteins from the E1 fraction that contained Cif activity (data not shown).

Our previous work had suggested that Cif was secreted, and size-exclusion chromatography demonstrated that Cif had a mass of >30 kDa (data not shown). Based on these previous findings, candidate proteins were prioritized based on size, the presence of secretory signals predicted by SMART (http://smart.embl-heidelberg.de/), and predicted localization

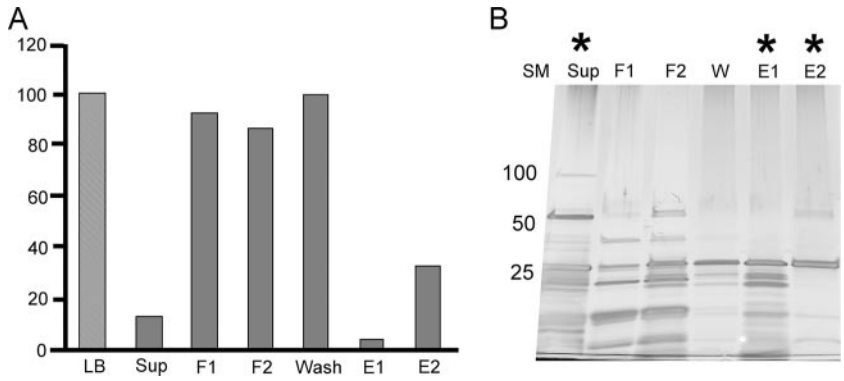

FIG. 1. Purification of Cif activity. (A) Apical membrane expression of CFTR following treatment of WT-MDCK cells with either LB medium (vehicle control), unfractionated culture supernatant (Sup), or flowthrough (F1 and F2), wash, and elution (E1 and E2) fractions. Percent apical membrane expression of CFTR was quantified as apical membrane expression of CFTR normalized to whole-cell lysate levels of CFTR relative to that of the LB control. (B) SDS-PAGE and silver stain analysis of crude supernatant and fractions from anion-exchange chromatography. Legend: SM, size markers; Sup, supernatant; F1 and F2, two flowthrough fractions; W, wash with 0 mM NaCl; E1, step elution with 50 mM NaCl; E2, step elution with 2 M NaCl. Asterisks indicates fractions with Cif activity.

(PSORT [http://psort.nibb.ac.jp/]). The top three candidate proteins, PA1914, PA2934, and PA4476, were selected for further study.

Single-crossover mutations were created in the PA1914, PA2934, and PA4476 genes, and the resulting mutants were assayed for the ability to decrease apical membrane expression of CFTR. Disrupting either the PA1914 or the PA4476 gene did not result in any loss of Cif activity (data not shown). Mutating the PA2934 gene with a single-crossover knockout mutation resulted in a loss of Cif activity (not shown), as did deletion of the PA2934 gene (Fig. 2A).

The PA2934 gene was cloned into the multicopy, arabinose-inducible plasmid pMQ70 alone (not shown) or with a hexahistidine tag fused to the carboxy terminus of the protein, resulting in plasmid pDPM73. Expression of the WT PA2934 protein from a multicopy plasmid (not shown) or of a His-tagged variant of PA2934 (pDPM73/PA2934-His) (Fig. 2A) was capable of complementing the mutation, thus demonstrating that a functional PA2934 protein is necessary for the previously reported Cif activity. The strain carrying the vector control had no effect on apical CFTR expression (data not shown). Furthermore, these data indicate that adding a His tag to the C terminus of PA2934 has no apparent impact on the function of the protein.

We next sought to determine if *E. coli*, which had previously been shown to lack Cif activity, demonstrated Cif activity when the PA2934-His protein was expressed in *trans* from a plasmid. Indeed, although *E. coli* had no Cif activity, when the PA2934-His protein was expressed in *E. coli* from an arabinose-inducible promoter on a multicopy plasmid, we observed that the resulting culture supernatants were capable of a small reduction in apical membrane expression of CFTR (Fig. 2B).

We also expressed Cif in *P. aeruginosa* PAO1, a strain previously shown to lack Cif activity (48), from plasmid pDPM73/PA2934-His. This strain reduced apical membrane expression ~20%, compared to the ~40% reduction observed for strain PA14. It is important to note that the basis for the lack of detectable Cif expression in the PAO1 strain is not known; for

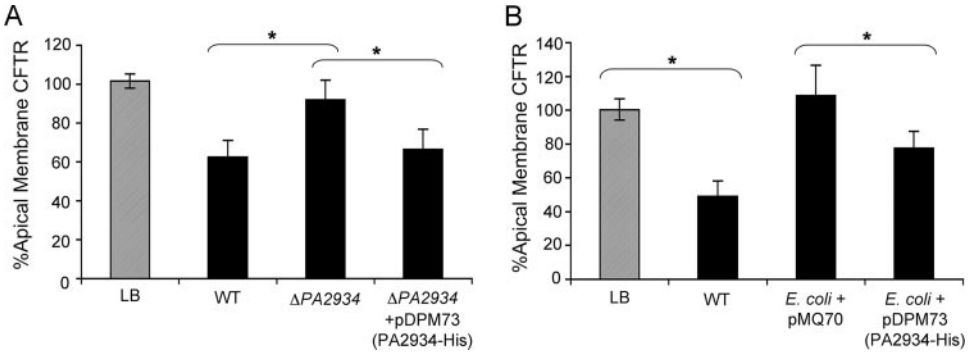

FIG. 2. PA2934 gene is necessary for Cif activity. (A) Apical membrane expression of CFTR in WT-MDCK cells exposed to LB medium or supernatant from either the WT, the ΔPA2934 mutant, or the ΔPA2934 mutant containing plasmid pDPM73 (PA2934-His), which expresses the His-tagged variant of the PA2934 protein. (B) Apical membrane expression of CFTR in WT-MDCK cells following incubation with LB medium or supernatant from WT *P. aeruginosa* PA14, *E. coli* Top10 carrying the empty vector pMQ70, or *E. coli* Top10 expressing the PA2934-His protein from plasmid pDPM73. For both panels, cultures were grown in LB medium for ~18 h and the WT-MDCK cells were exposed to supernatants for 10 min before assaying apical membrane CFTR levels. Percent apical membrane expression of CFTR was quantified as apical membrane expression of CFTR normalized to whole-cell lysate levels of CFTR relative to that of the LB control. Asterisks indicate statistically significant differences ($P < 0.05$).

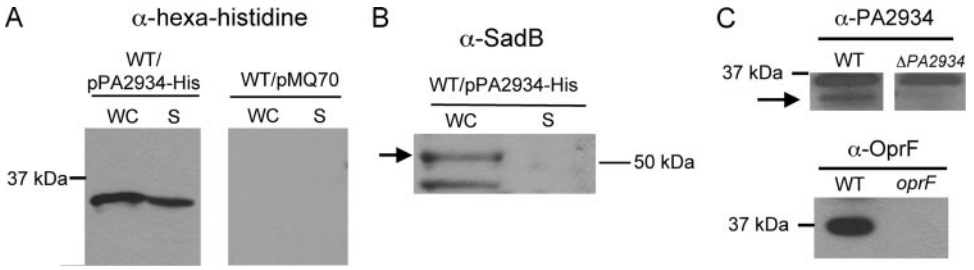

FIG. 3. PA2934-His is secreted. (A) Western blot analysis of the whole-cell pellet (WC) and cell-free supernatant (S) of *P. aeruginosa* PA14 carrying either the PA2934-His-expressing plasmid pDPM73 (pPA2934-His) or the empty vector pMQ70. Protein (2.5 μg) from each sample was resolved by SDS-PAGE, transferred to a membrane, and probed using a monoclonal antipolyhistidine antibody. (B) Western blot analysis of identical fractions to those from panel A from the *P. aeruginosa* strain carrying plasmid pDPM73 (pPA2934-His). Samples were probed with polyclonal anti-SadB antibodies. The arrow indicates SadB, and the lower band is a nonspecific cross-reacting band reported previously (9). (C) Vesicles were purified from the WT, ΔPA2934, and *oprF*::Tn*5*-B22 (*oprF*) strains as described in Materials and Methods and used in Western blot experiments. Samples were probed with either polyclonal anti-PA2934 (left) or polyclonal anti-OprF (right) antibodies. The arrow indicates the PA2934-specific band.

example, this strain may be defective in secretion of Cif rather than in its production.

It is noteworthy that Cif activity is somewhat variable, but whether this is the result of Cif protein expression or interaction with the host cell or is inherent in the assay itself is unclear. Thus, this activity can vary from highly robust, as demonstrated by the 90% reduction seen in Fig. 1A for the unfractionated supernatant, to fairly low levels (~20%). Figure 1A represents a single experiment from which the active fraction identifying Cif was obtained. The data presented in Fig. 2 and subsequent figures are the averages for a minimum of four individual experiments, with three replicates per experiment.

PA2934-His is a secreted protein. Assays for Cif activity indicated that PA2934 is a secreted protein (48). To confirm this prediction, we performed Western blot analysis of whole-cell and supernatant fractions of *P. aeruginosa* carrying a plasmid which expressed PA2934-His (pDPM73) or the vector-only control (pMQ70).

As shown in Fig. 3A, a cross-reacting band could be detected in both the whole-cell and supernatant fractions of the PA2934-His-expressing strain, but not the strain carrying the vector, when probed with anti-His-tag antibody. To confirm that the supernatant-localized PA2934-His was not a result of cell lysis, we demonstrated that SadB, a known cytoplasmically localized protein (9), was detected in the whole-cell fraction but not the supernatant fraction of the same WT/pPA2934-His-derived samples (Fig. 3B). These data indicate that the PA2934-His protein found in the supernatant was not due to cell lysis. Taken together with the predicted Sec secretion signal sequence at the N terminus of PA2934 and the activity assays presented above, our data indicate that PA2934 is indeed secreted.

PA2934 is associated with extracellular vesicles. Several recent reports have shown that *P. aeruginosa* and other microbes package extracellular signaling molecules and toxins in outer membrane-derived vesicles (OMVs) (12, 28, 36). Furthermore, enterotoxigenic *E. coli* has been shown to deliver a toxin to a eukaryotic host via its OMVs (28). Given that PA2934 is a secreted protein and impacts eukaryotic cell function, we tested the hypothesis that PA2934 might also be associated with extracellular vesicles.

In studies using PA2934-His expressed from a plasmid (not shown), we found PA2934-His in both the soluble fraction and the pellet after centrifugation of culture supernatants at 40,000 × *g*, conditions which are known to pellet OMVs (24).

We next investigated whether the WT PA2934 protein expressed in its native context is also associated with vesicles. Membrane vesicles were prepared from the WT and the ΔPA2934 mutant, as reported previously (12, 24), and the vesicle-containing fractions were probed with a polyclonal antibody to the PA2934 protein. Consistent with the localization of PA2934 protein to vesicles, a cross-reacting band migrating at the expected molecular mass of ~33 kDa was observed for the vesicle fraction of the WT, but not that of the ΔPA2934 mutant strain (Fig. 3C, top panel, arrow). Furthermore, the same fractions were probed with a polyclonal antibody to the outer membrane protein OprF, because vesicles have been shown to accumulate outer membrane proteins (43). A cross-reacting band of the expected size was detected in the vesicle fraction of the WT but not in that of the *oprF* mutant (Fig. 3C, bottom panel). These data indicate that PA2934 is likely associated with membrane vesicles.

Purified PA2934-His decreases apical membrane expression of CFTR. To demonstrate that PA2934 is both necessary and sufficient for Cif activity, we purified the PA2934-His protein from culture supernatants. The PA2934-His protein could be detected in crude cell-free supernatants (Fig. 4A) of an arabinose-induced *E. coli* strain expressing this protein from an arabinose-inducible promoter (pDPM73). Using the His-affinity resin, the PA2934-His protein was purified to apparent homogeneity from culture supernatants (Fig. 4A). The bands observed both in the crude supernatants and in fractionated samples correspond with the predicted size of Cif, at ~33 kDa.

When the purified PA2934-His protein was applied to WT-MDCK cells, it was capable of a time-dependent reduction of apical membrane expression of CFTR (Fig. 4B). By 60 min after treatment with PA2934-His, the apical membrane expression of CFTR was reduced >60%, and it was reduced >70% at 90 min. Similar results were obtained when PA2934-His was applied to the apical surfaces of WT-CFBE cells (Fig. 4C), indicating that the effects of PA2934-His on CFTR are not cell line dependent. These data demonstrate that PA2934 is both necessary and sufficient for the previously described Cif activ-

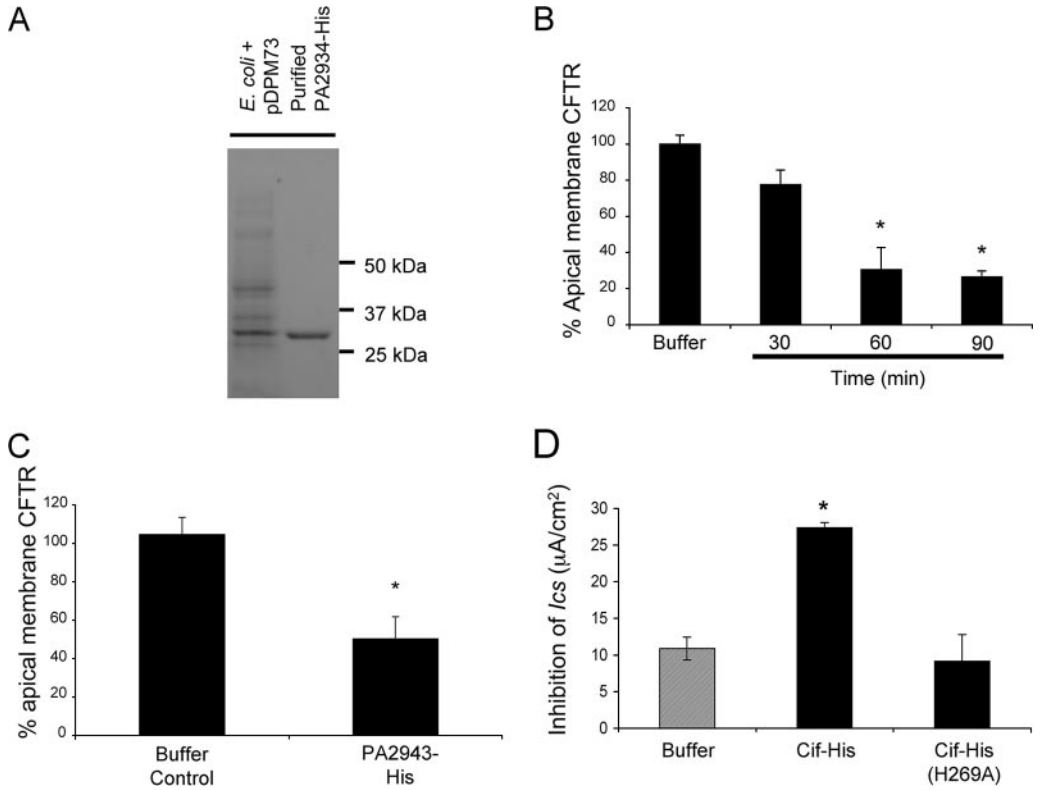

FIG. 4. PA2934 is sufficient for Cif activity. (A) Coomassie blue-stained SDS-PAGE gel of supernatant from *E. coli* Top10 cells carrying pDPM73 (PA2934-His) and of purified PA2934-His. Ten micrograms of protein from each sample was resolved via SDS-PAGE and stained using Coomassie brilliant blue. (B) Purified PA2934-His decreases apical membrane expression of CFTR in a time-dependent manner. Fifty micrograms of purified PA2934-His was added to the apical membranes of WT-MDCK cells and incubated for either 30, 60, or 90 min, followed by Western blot analysis to determine apical membrane expression of CFTR. (C) Apical membrane expression of CFTR in WT-CFBE cells was determined at 60 min as described for panel B. (D) Ussing chamber experiment to assay the effect of purified Cif-His protein or the Cif(H269A)-His mutant protein on CFTR-mediated Cl^- secretion in WT-CFBE cells. The forskolin-stimulated I_{sc} values were measured as described in Materials and Methods. I_{sc} data are expressed as changes in forskolin-stimulated I_{sc}. Asterisks indicate statistically significant differences from the buffer ($P < 0.05$).

ity. These findings have prompted us to rename the PA2934 gene and the resulting protein product *cif* and Cif, respectively, for *C*FTR *i*nhibitory *f*actor.

It should be noted that while the experiments presented here show percentages of apical CFTR normalized to whole-cell levels of CFTR, in previous studies we have normalized apical CFTR to ezrin and total cellular protein levels (48), clearly demonstrating that Cif activity decreases apical CFTR expression.

Cif inhibits CFTR-mediated chloride secretion. Our previous report demonstrated that the decrease in apical membrane expression of CFTR following treatment with Cif-containing culture supernatants was accompanied by a decrease in CFTR-mediated chloride secretion (48). In order to demonstrate that purified Cif was sufficient for both the decreased apical membrane expression of CFTR and the decrease in CFTR-mediated chloride secretion, we performed Ussing chamber experiments using the purified Cif-His protein.

Purified Cif-His protein added to the apical face of these cells inhibited forskolin-stimulated, CFTR-mediated Cl^- ion secretion by almost 30 $\mu A/cm^2$, whereas buffer alone had no significant effect on forskolin-stimulated CFTR Cl current (Fig. 4D). As a control, the addition of the specific CFTR inhibitor $CFTR_{inh}$-172 (32) to WT-CFBE cells reduced forskolin-stimulated, CFTR-mediated Cl^- ion secretion by almost 50 $\mu A/cm^2$ (not shown). Thus, recombinant Cif-His reduced CFTR Cl^- currents by ~60% and apical plasma CFTR Cl^- ion channel expression by approximately the same amount (~60%) (Fig. 4B).

Cif demonstrates epoxide hydrolase activity. Bioinformatic analysis suggests that the *cif* gene may belong to a three-gene operon including *morB*, encoding a predicted morphinone reductase, and the PA2933 gene, which is predicted to encode an MFS family transporter. The Cif protein is predicted to contain an α/β hydrolase fold. Besides the secretion signal, no other known domain was detected in the Cif protein. The α/β hydrolase superfamily is comprised of several subfamilies of proteins, including acyltransferases, lipases, lysophospholipases, and epoxide hydrolases (23). Further examination of the protein sequence suggested that Cif contains domains consistent with those of epoxide hydrolases, a diverse family of proteins associated with the degradation of xenobiotic compounds and involved in mammalian cell signaling.

To assess whether Cif acts as an epoxide hydrolase, we assessed its ability to degrade the synthetic epoxide hydrolase substrate (*S*)-NEPC. The assay used measures the production

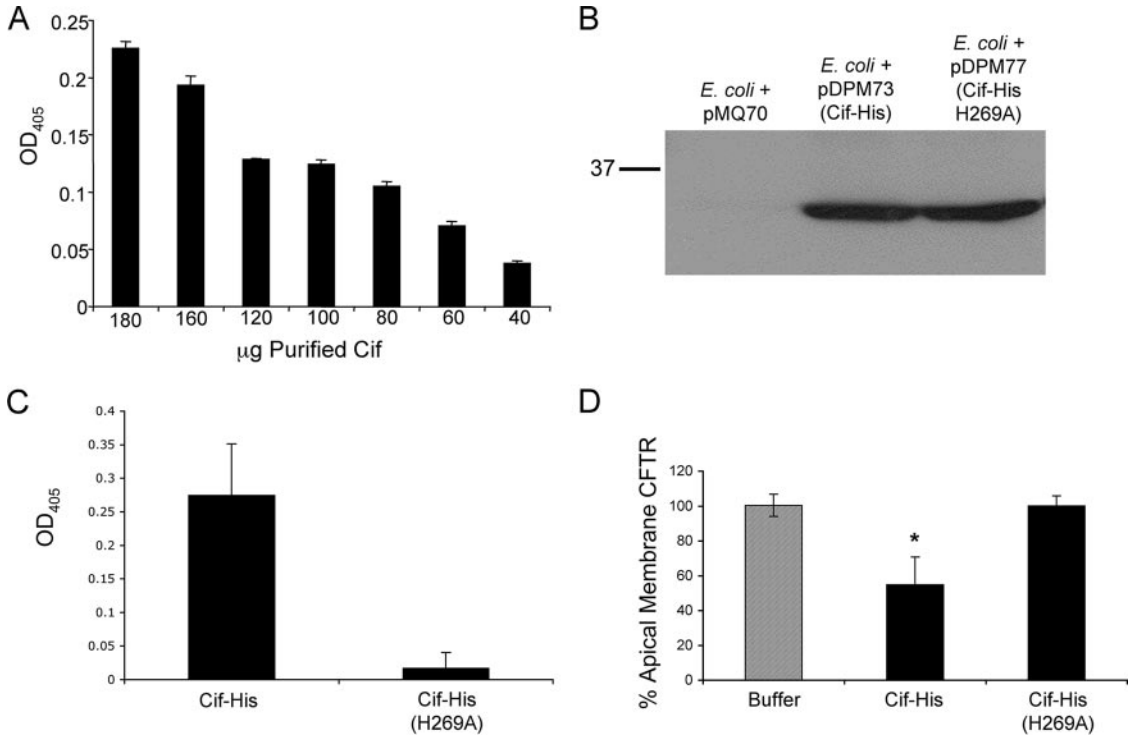

FIG. 5. Biochemical characterization of Cif. (A) Cif degrades the epoxide hydrolase substrate (*S*)-NEPC in a concentration-dependent manner. Various amounts of purified Cif-His, as indicated, were incubated with 100 μM of (*S*)-NEPC in 20 mM Tris, pH 8.5, and 500 mM NaCl for 20 min, and substrate degradation was determined by an increase in absorbance (OD_{405}). Results are reported as net enzyme-mediated substrate degradation. Spontaneous degradation, as measured by the absorbance of the substrate in the assay buffer lacking enzyme, was subtracted from the absorbance under experimental conditions. (B) Supernatants from *E. coli* cultures carrying either the empty vector (pMQ70) or a plasmid expressing Cif-His (pDPM73) or the Cif(H269A)-His mutant (pDPM77) were harvested via centrifugation, filtered, resolved by SDS-PAGE, and probed using a monoclonal antihexahistidine antibody. (C) The Cif(H269A)-His mutant protein is incapable of degrading (*S*)-NEPC. One hundred micrograms of either the purified Cif-His protein or the Cif(H269A)-His mutant protein was assayed for the ability to degrade (*S*)-NEPC, as described above. (D) Fifty micrograms of either purified WT Cif or the purified Cif(H269A)-His mutant protein was added to the apical membranes of WT-MDCK monolayers. Apical membrane expression of CFTR was assayed via Western blotting and normalized to whole-cell lysate CFTR.

of the product *p*-nitrophenol, which is yellow and absorbs maximally at 405 nm. As shown in Fig. 5A, purified Cif was capable of degrading this compound in a protein concentration-dependent manner during the 60-min incubation period of the assay. Control experiments demonstrated that the Cif protein does not absorb at 405 nm, and thus the increase in absorbance was due to degradation of the substrate.

Cif hydrolase activity requires the invariant histidine residue. Members of the α/β hydrolase family, including typical epoxide hydrolases, are characterized by the presence of a catalytic triad consisting of a nucleophilic residue, an acidic residue, and a highly conserved histidine, located within three separate loops of the protein (23). Examination of the amino acid sequence of Cif identified these residues, including the invariant histidine residue. We created a mutant variant of Cif in the purification vector, wherein the histidine residue at position 269 was replaced with an alanine residue. Western blot analysis of *E. coli* expressing either Cif-His or the Cif(H269A)-His variant demonstrated that the H269A variant was stably expressed (Fig. 5B).

The Cif(H269A)-His protein was purified to apparent homogeneity by the same method described for the WT Cif-His protein and then assayed for epoxide hydrolase activity as described above. The H269A mutation significantly reduced the epoxide hydrolase activity of this variant compared to that of the WT protein, resulting in activity at or near the levels seen for the buffer control (Fig. 5C).

Loss of epoxide hydrolase activity correlates with a loss of Cif activity. To correlate epoxide hydrolase activity with the previously described Cif activity, we tested the ability of the purified H269A variant of Cif to decrease apical membrane expression of CFTR. The WT Cif-His protein significantly decreased the apical membrane expression of CFTR, but the Cif(H269A)-His mutant protein had no effect on the apical membrane expression of CFTR in WT-MDCK cells (Fig. 5D). Similarly, the Cif(H269A)-His mutant protein had little or no effect on CFTR-mediated Cl^- secretion in WT-CFBE cells (Fig. 4D). These data, along with the observation that the H269A mutant lacks epoxide hydrolase activity, suggest that epoxide hydrolase activity is linked to Cif activity.

***cif* gene expression is higher in nonmucoid clinical isolates than in mucoid clinical isolates.** To better understand the potential clinical relevance of Cif activity, we sought to characterize *cif* gene expression in *P. aeruginosa* clinical isolates, specifically those isolates that originated from CF patients. To do this, we first divided the clinical isolates from CF patients

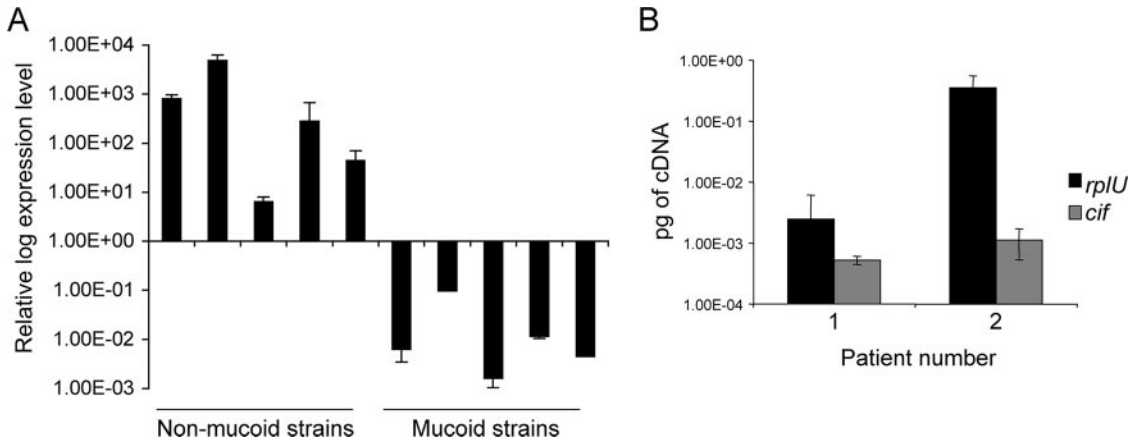

FIG. 6. Expression of *cif* in clinical isolates and in CF sputum. (A) qRT-PCR analysis of *cif* gene expression in WT *P. aeruginosa* PA14 and in mucoid and nonmucoid *P. aeruginosa* clinical strains isolated from CF sputa. Expression levels were quantified as picograms of input cDNA and normalized to *rplU* expression. Values are relative to the *cif* gene transcript level observed for *P. aeruginosa* PA14 (arbitrarily set to 1). (B) qRT-PCR analysis of *cif* and *rplU* expression levels in RNAs purified from the sputa of CF patients. Expression levels were quantified as picograms of input cDNA based on a standard DNA curve.

into either mucoid or nonmucoid strains, based on their phenotypes on solid LB medium (data not shown). Nonmucoid strains are typically associated with early infection of the CF lung, while mucoid strains are associated with chronic lung infections (13, 14, 35).

To quantitatively assess *cif* gene expression, strains were grown in LB medium to an OD_{600} of ~2.5, the total cellular RNA was harvested, and qRT-PCR was performed. Interestingly, we found that expression of the *cif* gene was significantly higher in all five nonmucoid clinical isolates than in *P. aeruginosa* PA14 (set arbitrarily at 1 in this experiment) and the mucoid *P. aeruginosa* strains (Fig. 6A).

The *cif* gene is transcribed in *P. aeruginosa* cells growing in the CF lung. To investigate whether *cif* might play a role in the context of the CF lung, we tested whether the population of *P. aeruginosa* cells residing in sputum within the CF lung expressed this gene. Total RNAs were purified from the sputa of two CF patients, cDNAs were synthesized, and the expression of the *cif* gene was assessed by qRT-PCR. As shown in Fig. 6B, the *cif* gene transcript was indeed detectable in the sputum samples of both patients tested, as was the constitutively expressed *rplU* gene, suggesting that *cif* is transcribed in the CF airway. It should be noted that expression levels of the *rplU* and *cif* genes for both of the patients tested were several orders of magnitude higher than those for the no-reverse-transcriptase controls. Furthermore, the transcript level for each gene and the relative expression pattern seen for these two patients, with *rplU* expression exceeding *cif* expression, were similar to those observed for the mucoid clinical isolates described above.

DISCUSSION

We previously demonstrated that *P. aeruginosa* secretes a factor capable of decreasing the apical membrane expression of both WT and ΔF508 CFTR (48). Here we continued this work, first purifying and identifying the factor as the product of the PA2934 gene and then demonstrating both the necessity and sufficiency of this gene (*cif*) and its product (Cif) for the previously described activity. We show that Cif both decreases apical CFTR expression and reduces CFTR-mediated Cl^- ion secretion, indicating that Cif activity has a functional impact on the biology of airway cells relevant to CF.

Our data show that Cif is a secreted protein and that supernatants of *P. aeruginosa* are sufficient to observe Cif activity. Therefore, direct contact of the bacterium with the airway cells is not necessary for Cif to exert its effects on these cells. Furthermore, our data show that Cif is associated with membrane vesicles, indicating that fusion of the diffusible vesicles with epithelial cells may be one mechanism by which Cif enters the cytosol of eukaryotic cells (24). Recent data support the idea that biofilms of *P. aeruginosa* in the CF lung may form in the mucus above the airway cells rather than directly on the airway cells (51). However, given that Cif is secreted and likely diffusible in the context of the CF lung and that purified Cif protein in the absence of bacteria is sufficient to decrease apical membrane CFTR expression, this toxin would still be capable of impacting airway cell biology in CF even if expressed from bacteria in the mucus.

It is widely accepted that bacteria are capable of altering trafficking in eukaryotic cells. For example, both *Mycobacterium tuberculosis* and *Legionella pneumophila* rely on their ability to arrest phagosomal maturation during infection of macrophages (4, 44, 49). While the alteration of trafficking is relatively commonplace among intracellular pathogens, it is not typically associated with extracellular pathogens such as *P. aeruginosa.* Therefore, Cif-mediated alteration of CFTR trafficking may represent a novel mechanism of altering the cell biology of the host by an extracellular pathogen. We speculate that the alteration of protein localization following secretion of Cif creates an environment conducive for *P. aeruginosa* colonization. That is, in the context of CF, Cif may further reduce any residual Cl^- ion secretion and airway surface liquid volume and thereby further reduce mucociliary clearance, allowing establishment of the *P. aeruginosa* infection and, eventually, biofilm formation.

We demonstrated that Cif can catalyze the degradation of a

model substrate of epoxide hydrolases, (*S*)-NEPC, which was demonstrated previously to be a substrate of a murine epoxide hydrolase (15). Both the primary amino acid sequence of Cif and its predicted secondary structure place this protein within the broad family of α/β hydrolase enzymes. Interestingly, proteins in this family share a conserved α/β fold motif as well as a conserved catalytic triad. Consistent with Cif's identification as a member of the α/β hydrolase family, we have shown that mutating the invariant histidine residue predicted to comprise the conserved catalytic triad, i.e., His269, to Ala results in a loss of epoxide hydrolase activity.

Many of the members of the α/β hydrolase family are considered promiscuous based on their ability to degrade a number of different ester-based bonds with various affinities (23). Thus, while Cif is capable of hydrolyzing a model epoxide substrate in vitro, the decreased apical membrane expression of CFTR mediated by Cif in vivo may result from this enzyme acting on a very different substrate. Identifying the authentic substrate for this protein is the subject of ongoing studies.

The expression and secretion of epoxide hydrolases by pseudomonads have been reported previously. Work by Jacobs et al. identified a protein in culture supernatants of a soil pseudomonad capable of degrading the epoxide epichlorohydrin (26). Interestingly, a partial sequence of ~50 amino acids of this epichlorohydrin hydrolase showed 40% identity and 62% similarity to Cif (PA2934) of *P. aeruginosa*. It was the similarity of PA2934 to this reported epichlorohydrin hydrolase that prompted us to test Cif for this activity.

The demonstration that the *cif* transcript is detectable within CF sputum suggests that this gene, and perhaps its protein product, may be expressed in the CF airway and thus may contribute to the pathogenesis of *P. aeruginosa* in this context. It should be noted that common CF airway pathogens, including *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, and *Haemophilus influenzae*, apparently lack the *cif* gene, based on BLAST analysis. Interestingly, a potential homolog sharing ~38% amino acid sequence identity with Cif was observed in *Burkholderia cepacia*. Therefore, it is possible that the *cif* transcript detected might reflect combined expression of this gene from *P. aeruginosa* and *B. cepacia*. Furthermore, the data demonstrating that *cif* gene expression is relatively high in nonmucoid isolates, which are associated with early colonization of the CF lung, and relatively low in mucoid isolates, which are associated with the long-term chronic colonization of the CF lung, suggest that *cif* expression may be temporal in nature (13, 14, 35). Specifically, *cif* gene expression may be important early in the colonization of the CF lung but may play little or no role in later chronic infection processes.

The data above prompted us to propose a two-stage model of CF lung infection in which Cif plays a key role in the initial stage of infection, perhaps by further reducing CFTR expression in CF patients and thus facilitating subsequent biofilm formation by *P. aeruginosa*. In this model, *P. aeruginosa* enters the lung and may be able to gain an initial foothold due to the decreased expression of CFTR in a CF patient and the associated decrease in mucociliary clearance and other innate resistance pathways. *P. aeruginosa* strains expressing Cif may have a selective advantage in continued persistence in the lung because these organisms can further decrease the already depleted levels of apical membrane CFTR, thus resulting in the inability of the lung to clear this microbe. As lung function decreases, the bacterial infection progresses, leading eventually to biofilm formation and the mucoid phenotype, wherein Cif function is no longer required and therefore there may be a selective advantage in down regulating its expression. We believe that this model could account for why *P. aeruginosa* is particularly suited to infect the CF lung.

Much of the focus in the area of CF therapeutics has centered on discovering compounds capable of increasing apical membrane expression of the ΔF508-CFTR protein. The rationale underlying such a strategy is that increased CFTR activity and increased Cl^- ion secretion would result in decreased mucous viscosity, increased mucociliary clearance, and resolution of infections and symptoms. The work presented here and our earlier work suggest that in the presence of *P. aeruginosa*, as is the case for the majority of CF patients, this treatment course might be ineffectual, as any increase in ΔF508-CFTR protein would be reversed due to the activity of Cif. Thus, any attempt to increase apical membrane expression of ΔF508-CFTR may need to be coupled to either an antipseudomonas regimen of antibiotics or compounds specifically targeted to Cif function.

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