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 cystic fibrosis transmembrane conductance regulator (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 CFTR inhibitory factor. 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* during their lifetime, and this organism is the leading cause of morbidity and mortality among CF patients (5, 17). In roughly 90% of individuals suffering from CF become infected with *P. aeruginosa* during their lifetime, and this organism is the leading source 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 (CFTR inhibitory factor) (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*)
<table>
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<tr>
<th>Strain, plasmid, or primer</th>
<th>Relevant genotype, description, or sequence (5’–3’)</th>
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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 Centricon prepurification filters per the manufacturer’s instructions (Millipore, Billerica, MA). Concentrated supernatants were dialyzed against 4 liters of 25 mM morpho-
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**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 Kek Proteomics and Mass Spectrometry facility at Yale University for MudPIT analy-
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OD$_{560}$ 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 for each fraction were precipitated with 10% trichloroacetic acid (TCA) and visualized 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 spuata 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. cDNAs were analyzed spectrophotometrically to determine their concentration, and the absence of DNA was assessed by real-time RT-PCR analysis.

Vesicle fractionation. Vesicles were purified from an overnight culture of P. aeruginosa strain PA14 reduced 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 unfractonated 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

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).
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

![](image)

FIG. 1. Purification of Cif activity. (A) Apical membrane expression of CFTR following treatment of WT-MDCK cells with either LB medium (vehicle control), unfraccionated 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.

![](image)

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).
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 unfraccionated 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 strain, when probed with anti-His-tag antibody. 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-
ity. These findings have prompted us to rename the PA2934 gene and the resulting protein product cif and Cif, respectively, for CFTR inhibitory factor.

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 μA/cm², 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 CFTRinh-172 (32) to WT-CFBE cells reduced forskolin-stimulated, CFTR-mediated Cl⁻ ion secretion by almost 50 μA/cm² (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 epoxide hydrolase fold. Besides the secretion signal, no other known domain was detected in the Cif protein. The epoxide 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
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 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 mutant 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...
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\textsubscript{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\textsuperscript{-} 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\textsuperscript{-} 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, Serratia marcescens, 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 deleterious 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 ineffective, 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 antisipueudosmas regimen of antibiotics or compounds specifically targeted to Cif function.

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