Signature Motifs Identify an Acinetobacter Cif Virulence Factor with Epoxide Hydrolase Activity*

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‡The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; Cif, CFTR inhibitory factor; EH, epoxide hydrolase; qRT, quantitative real-time.

Endocytic recycling of the cystic fibrosis transmembrane conductance regulator (CFTR) is blocked by the CFTR inhibitory factor (Cif). Originally discovered in Pseudomonas aeruginosa, Cif is a secreted epoxide hydrolase that is transcriptionally regulated by CifR, an epoxide-sensitive repressor. In this report, we investigate a homologous protein found in strains of the emerging nosocomial pathogens Acinetobacter nosocomialis and Acinetobacter baumannii (“aCif”). Like Cif, aCif is an epoxide hydrolase that carries an N-terminal secretion signal and can be purified from culture supernatants. When applied directly to polarized airway epithelial cells, mature aCif triggers a reduction in CFTR abundance at the apical membrane. Biochemical and crystallographic studies reveal a dimeric assembly with a stereochemically conserved active site, confirming our motif-based identification of candidate Cif-like pathogenic EH sequences. Furthermore, cif expression is transcriptionally repressed by a CifR homolog (“aCifR”) and is induced in the presence of epoxides. Overall, this Acinetobacter protein recapitulates the essential attributes of the Pseudomonas Cif system and thus may facilitate airway colonization in nosocomial lung infections.

Opportunistic lung infections by Gram-negative pathogens are a major source of mortality and morbidity. In patients with cystic fibrosis (CF),2 airway mucociliary transport is impaired by loss of function in the cystic fibrosis transmembrane conductance regulator (CFTR), an ion channel essential for proper mucus hydration (1). The resulting breakdown of airway clearance permits colonization by bacteria such as Pseudomonas aeruginosa and Burkholderia cepacia complex, leading to cycles of infection and inflammation that impair respiratory function and lead ultimately to death (2). Smoking also suppresses airway epithelial CFTR (3–6) and contributes to the etiology of chronic obstructive pulmonary disease, the third leading cause of mortality in the United States (7). Although the role of P. aeruginosa in chronic obstructive pulmonary disease is complex, it has been associated with exacerbations, reduced lung function, and increased mortality (8–11). Finally, Acinetobacter species cause drug-resistant nosocomial infections in the lung and elsewhere (12, 13), and include one of the clinically significant “ESKAPE” pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa, and Enterobacter species) (14, 15).

In addition to exploiting weakened host immunity, these pathogens deploy a variety of highly sophisticated virulence factors with more specialized targets. Among these is the CFTR inhibitory factor (Cif), a recently characterized P. aeruginosa protein expressed both in the PA14 strain and in clinical isolates (20, 21). Originally characterized as an extracellular factor that inhibited CFTR-mediated chloride currents across polarized epithelial monolayers (22), Cif is both directly secreted and is also packaged into outer membrane vesicles that dramatically enhance delivery to airway epithelial cells (23). Further investigation revealed that Cif acts by triggering the post-endocytic degradation of CFTR, reducing its cell-surface abundance (22). As a result, Cif has the potential to interfere with mucociliary clearance mechanisms, in concert with previously identified Pseudomonas ciliostatic virulence factors, such as pyocyanin, rhamnolipids, and phenazines (24–26).

Sequence and structural homologies indicate that Cif adopts an αβ hydrolase-fold, a scaffold commonly associated with hydrolytic enzymes (20, 27). Based on alignments to other αβ hydrolase family members, it was originally proposed to act as

Background: Pathogens target airway clearance mechanisms to facilitate infection.
Results: Sequence analysis reveals an Acinetobacter epoxide hydrolase (EH) that triggers loss of the cystic fibrosis transmembrane conductance regulator (CFTR).
Conclusion: Homologous EH virulence factors found in a variety of opportunistic pathogens can impair CFTR, a key element of host airway defenses.
Significance: EH virulence factors are potential therapeutic targets.
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**TABLE 1**

| Primers used | Sequence (5’-3’) |
|--------------|-----------------|
| RBS/13TU cif F | 5’-TAAGGAGTTAGGAGGTTGAGAGAAAGCAATAGAAATTTTTTTAAAATTTTACCTTT-3’ |
| His6/13TU cif R | 5’-ATTGAGAGGTTGAGAGAAAGCAATAGAAATTTTTTTAAAATTTTACCTTT-3’ |
| 70/RBS S | 5’-TGTTTTCTCCATATGCGGTTTGGGTCAGAGATTCGGAGCTAGAATGAAAGAGACATG-3’ |
| 70/His6 Y | 5’-TGATGGAGGTTGAGAGAAAGCAATAGAAATTTTTTTAAAATTTTACCTTT-3’ |

**Cloning Acinetobacter cif for recombinant expression**

**Deletion of Acinetobacter cifR**

| cifR 1 | 5’-GAGCTGTTAAATTTCTCTCCACGCGCAAACCTTCTCCTCGTTCTGCCACGAAATTATTTATAAAGTT-3’ |
| cifR 2 | 5’-GATATAAAATTGAGACGAAATTAAAAAAATTATTTATTTATAGTTAGTAAATACATTGTTTTGTTCTTACCTTTAAATTT-3’ |
| cifR 3 | 5’-CACTTAATAAAACATTTAGCCTCCTCTCCTTTGTTATTTTATACACTTTGAAAAACACAG-3’ |
| cifR 4 | 5’-GCGAGTAACGTTTTCACTACGGAACAAAATTTTCTTTGAGTTCTGGACTAAACGG-3’ |

**Quantitative Real-time PCR**

| 13TU cifR | 5’-GCGGAAATGGTTATGAAGCGACG-3’ |
| 13TU cifF | 5’-AAAGCCGCGAAAATCAACGTGCAGTTAC-3’ |
| 13TU rplL F | 5’-TGTTCTCCAGTTGTTGAGCCTAAAGTA-3’ |
| 13TU rplL R | 5’-TGACGCGTGGACCTTTGTGTGATTTCAACT-3’ |

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Based on similarity to the cif gene of *P. aeruginosa*, the coding region of NCBI accession number WP_004885628 (formerly ZP_05823503) from *A. nosocomialis* strain RUH2624 (30) was amplified with primers RBS/13TU cif F and 6-His/13TU cif R (Table 1) using Phusion polymerase (New England Biolabs). These primers added a canonical Shine-Dalgarno sequence and a carboxyl-terminal His6 tag to the gene for recombinant protein expression and purification from *Escherichia coli*. The resulting PCR product was purified using a Qiaquick PCR Purification Kit (Qiagen) and served as the template for a second round of PCR using the 70/RBS 5’ and 70/His6 3’ primers to add pMQ70 plasmid overlap regions to the ends. This final PCR product was cloned into plasmid pMQ70 via *Saccharomyces cerevisiae* recombineering as previously described (31).

Positive clones were verified by PCR and Sanger sequencing. Mutagenesis to generate the aCif-D158S mutant construct was performed using the QuickChange Lightning Site-directed Mutagenesis Kit (Stratagene), which was again verified by Sanger sequencing.

**Deletion of the Acinetobacter cifR Homolog**—An in-frame deletion of the cifR gene from the chromosome of *A. nosocomialis* strain RUH2624 was created using construct pMQ30-cifR. First, the pMQ30-cifR plasmid was created using *S. cerevisiae* recombineering (31), empty pMQ30 plasmid, and the primers listed in Table 1. The pMQ30-cifR construct was electropropared into *A. nosocomialis* using the procedure described by Choi et al. (32) for *P. aeruginosa*. Exconjugants containing an inserted plasmid were selected on LB agar supplemented with 50 µg/ml of gentamicin, followed by counterselection with 5% (w/v) sucrose. In-frame deletions were confirmed by colony PCR.

**Protein Purification**—Recombinant carboxyl-terminal His6*-tagged* aCif protein was expressed and secreted from *E. coli*, and purified by immobilized metal affinity chromatography using procedures previously described (33) for Cif.

**Biochemical Characterization**—Size-exclusion chromatography was carried out with a Superdex 200 HR 10/30 column (GE Healthcare) calibrated with aldolase, ovalbumin, chymotrypsigen A, and RNase A. The size-exclusion chromatography buffer consisted of 100 mM NaCl and 20 mM HEPES (pH 7.4). Circular dichroism (CD) spectroscopy was performed with a 1-mm path length quartz cuvette containing 300 µl of 10 µM aCif protein in 100 mM NaCl, 20 mM HEPES (pH 7.4). Edman degradation was performed by the W. M. Keck Biotechnology Resource Laboratory (Yale University) using purified aCif protein.

**EH Activity**—Epoxide hydrolysis activity was first assessed for a panel of candidate epoxides using an adrenochrome reporter end-point assay as described previously (27). Briefly, samples were incubated for 1 h at 37 °C in a 100 µl volume...
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containing 20 μM aCif in 100 mM NaCl with 20 mM MOPS (pH 7.4 at RT) and 1% (v/v) dimethyl sulfoxide. Next, 50 μl of 2 mM NaIO₄ in 90% (v/v) acetonitrile were added, and samples were incubated at room temperature for 1 h. A colorimetric signal was generated by addition of excess adrenaline-HCl and quantified by absorbance at 490 nm (A₄₉₀).

To determine specific activities, epoxides were incubated with varying aCif concentrations for 10 min at 2% (v/v) dimethyl sulfoxide. Samples were then incubated with 4 mM NaIO₄ for 30 min. To test the effect of phosphate, a parallel experiment was performed at the same assay pH with 20 mM sodium phosphate buffer in place of MOPS.

To quantitate product formation, standard curves were generated using 5 μM aCif and 0–2 mM of the cognate diols. After verifying linearity of the A₄₉₀ read-out in this range, various concentrations of aCif were incubated with 2 mM epoxide substrate under the conditions listed above, and specific activity was calculated in a range where the signal varied linearly with enzyme concentration. The amount of product formed was determined by linear regression analysis (Excel) of the standard curves.

Protein Crystallization and Data Collection—Purified aCif protein at 5 mg/ml in 100 mM NaCl, 20 mM HEPES (pH 7.4) was submitted to the Hauptman-Woodward Medical Research Institute High-Throughput Screening Facility for microbatch crystallization (34). Next, promising conditions were adapted to the hanging drop vapor diffusion method to obtain diffraction-quality protein crystals (33). aCif protein was mixed in a 1:1 ratio with well solution to achieve a total volume of 4 μl. The well solution consisted of 100 mM KH₂PO₄, 100 mM sodium citrate (pH 4.0), 20% (w/v) polyethylene glycol (PEG) 4000. Prior to data collection, crystals were harvested into a cryoprotectant solution consisting of 100 mM KH₂PO₄, 100 mM sodium citrate (pH 4.0), 20% (w/v) PEG 4000, 20% (w/v) glycerol, and flash cooled by plunging into a liquid nitrogen bath. Oscillation data were collected at 100 K at the X6A beamline of the National Synchrotron Light Source at Brookhaven National Laboratory. Diffraction images were processed and scaled using the XDS package (35). Phases were determined by molecular replacement. The search model was generated by threading the aCif protein sequence into the experimentally determined structure of Cif (PDB code 3KD2) using SWISS-MODEL (36, 37). Molecular replacement searches, model building, and iterative rounds of refinement were performed using Phenix (38, 39). Manual adjustment of the model was performed using WinCoot (40), and structural images were generated using PyMOL (41).

Protein crystals for aCif-D158S were obtained by the same method used for the wild-type (WT) protein, using a well solution consisting of 250 mM KH₂PO₄, 100 mM sodium citrate (pH 4.0), 20% (w/v) PEG 4000, and a cryoprotectant solution consisting of 250 mM KH₂PO₄, 100 mM sodium citrate (pH 4.0), 20% (w/v) PEG 4000, and 20% (w/v) glycerol. The aCif-WT model (PDB entry 4MEA) was used as a molecular replacement search model to obtain phase information, and all other data collection and processing steps were performed as for aCif-WT.

Cell Culture and CFTR Biotinylation Assay—The determination of apical membrane CFTR abundance was performed using CFBE4lo- cells stably transduced with WT-CFTR (CFBE-WT) (42). The effects of Cif virulence factors on CFTR levels were monitored after applying purified Cif or aCif protein to the apical surface of polarized cells, followed by domain-selective cell-surface biotinylation using EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce), pull-down using streptavidin-conjugated agarose, and quantitative Western blot for CFTR, as described previously (20, 43, 44).

RNA Isolation, cDNA Synthesis, and qRT-PCR—Acinetobacter nosocomialis strain RHH2624 was cultivated overnight at 37 °C in LB and subcultured 1:100 into 5 ml of LB with 1 mM epibromohydrin, (R)-styrene oxide, or (S)-styrene oxide as indicated. Cultures were grown to an A₆₀₀ of 1.0, upon which 1 ml was removed and centrifuged briefly to pellet cells. The pellets were flash-frozen in a dry ice-ethanol bath and stored at −80 °C. RNA was isolated as described previously (21).

cDNA was synthesized from 1 μg of purified RNA using the Quantitect Reverse Transcription Kit (Qiagen). Quantitative real-time (qRT) PCR primers were designed to the Acinetobacter nosocomialis genes cif (13TU cifF and 13TU cifR) and cifR (13TU CifR F and 13TU CifR R) as a transcriptional control (GenBank™ accession numbers: EEX01162, EEX01160, and EEX01619, respectively; Table 1). Primers were tested for specificity by PCR of the genomic DNA. qRT-PCR was performed with SYBR Green PCR master mix (Applied Biosystems) using a CFX96 Real-Time system on a C1000 thermal cycler (Bio-Rad) for 42 cycles, followed by a melt curve to verify proper amplification. Real-time data were analyzed using CFX manager software (Bio-Rad) and expressed as picograms of input RNA of the gene of interest relative to the ribosomal transcriptional control.

RESULTS AND DISCUSSION

aCif Exhibits Cif-like Epoxide Hydrolase Activity—The putative aCif proteins from Acinetobacter nosocomialis (GenBank locus EEX01162) and Acinetobacter baumannii (locus EKU60908) exhibit 100% sequence identity to each other. To begin our investigation of the aCif protein, we first performed a full sequence alignment with the Cif protein from P. aeruginosa (Fig. 1). aCif contains a 24-residue N-terminal sequence predicted to act as a secretion signal by SignalP (45), corresponding to the validated signal sequence observed in Cif (33). In aCif, this sequence is followed by a 27-residue linker, and then by a 298-residue region with 36% sequence identity to the mature Cif sequence (Fig. 1).

As a basis for biochemical characterization, we cloned the cif gene from Acinetobacter nosocomialis into the pBAD arabinose-inducible expression vector pMQ70 with a carboxyl-terminal His₆ tag. Recombinant aCif could be readily purified from culture supernatants of E. coli transformed with the expression vector. Furthermore, Edman sequencing of the amino terminus of purified aCif yielded a sequence of lyEDPNLKSIDT (numbered relative to the predicted translational start site), consistent with cleavage of the predicted signal sequence during secretion. The mature aCif protein thus carries a 27-residue extension at the N terminus of the Cif homology domain.
Cif has previously been shown to form a dimer in solution (27). To test the oligomeric state of aCif, we calibrated the elution volume of aCif during analytical size-exclusion chromatography relative to a series of mass standards. The elution volume of 14.95 ml corresponds to a molecular mass of 60 kDa (Fig. 2A), substantially larger than the predicted monomeric molecular mass of 37 kDa, and directly comparable with the estimate obtained previously for Cif using analytical size-exclusion chromatography and sedimentation analysis. The protein is also thermally stable, with a melting temperature of 51.2 °C (Fig. 2B), consistent with the requirement that it function in the potentially challenging environment outside the cell.

To test the functional similarity of the two proteins, we sought to determine whether aCif possesses EH activity. Using an adrenochrome reporter assay, we measured the ability of aCif to catalyze vicinal diol formation from a series of candidate epoxide substrates (Fig. 3A). The strongest signals were observed with three compounds: epoxycyclohexane and the \((S)\)-stereoisomers of styrene oxide and 4-nitrostyrene oxide. We next determined specific activities for aCif as a catalyst of hydrolysis for these compounds and for \((R)\)-styrene oxide, which showed a lower signal in the adrenochrome assay (Fig. 3A). Compared with controls, statistically significant specific activities were observed for the three expected substrates, but not for \((R)\)-styrene oxide (Fig. 3B). Taken together, these data clearly demonstrate that aCif can catalyze the hydrolysis of multiple epoxide compounds. We therefore conclude that, like the Cif protein from \(P.\) \(aeruginosa\), aCif is a \textit{bona fide} EH.

We also calculated a background threshold for the adrenochrome signal based on the confirmed lack of aCif activity for \((R)\)-styrene oxide (Fig. 3A). Aside from the three substrates tested in Fig. 3B, no other epoxide showed a level of hydrolysis significantly above this background, suggesting that none of them is a substrate. Thus, the aCif active site exhibits a high level of stereoselectivity.

Interestingly, we were unable to detect significant aCif catalysis of epibromohydrin, a substrate hydrolyzed by \(P.\) \(aeruginosa\) Cif and originally used to characterize its EH activity (27, 46). Direct side-by-side epibromohydrin hydrolysis assays confirmed the discrepancy (data not shown). The residues surrounding the catalytic machinery of aCif are remarkably conserved with Cif, in both identity and placement, consistent with a shared enzyme activity. However, the residues that line the entrance to the active site display more significant divergence, including backbone location as well as residue identity. Furthermore, position of Cif residue His-269, which was previously
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FIGURE 3. aCif is a Cif-like EH virulence factor. A, the epoxide hydrolysis ability of aCif was examined for a small panel of epoxide substrates; gray shading indicates the signal obtained with (R)-styrene oxide. B, the specific activity of hydrolysis of aCif was determined for four epoxides using a calibrated adrenochrome assay in MOPS buffer, or at (right) in sodium phosphate buffer at the same pH. C, 50 μg of Cif or aCif protein were applied apically to polarized human airway epithelial cells for 60 min at 37 °C, and cell-surface CFTR levels were determined by SDS-PAGE and immunoblot analysis of surface-biotinylated proteins. Values shown are mean ± S.D. (panel A) or mean ± S.E. (panels B and C) (*, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., not significant; n ≥ 3).

postulated to play a role in substrate selectivity determination (33), is occupied by Glu-25, the N-terminal residue of mature aCif, suggesting that the N-terminal extension may participate in substrate selection. Thus, differential active-site accessibility may provide one explanation for observed discrepancies in the substrate profiles for these two homologous Cif EHs.

aCif Can Reduce CFTR Apical Membrane Levels—The physiological activity that led to the identification of Cif was a decrease in cell-surface CFTR abundance in epithelial cells co-cultured with P. aeruginosa (22). This CFTR effect does not require the presence of live bacteria. It can be mediated directly by purified protein, which permitted the initial isolation of Cif and the proteomic identification of Cif as the causative agent (20, 27). Given the different specificity profiles of Cif and aCif, we wished to determine whether aCif could also trigger a reduction in CFTR at the apical membrane. We applied aCif or Cif to the surface of polarized human bronchial epithelial cells that were grown in culture at an air-liquid interface, and monitored cell-surface CFTR abundance by biotinylation, streptavidin capture, and immunoblotting. Both aCif and Cif exhibit a robust ability to reduce the levels of CFTR at the cell surface (Fig. 3C), with similar efficacies. These data clearly demonstrate that aCif recapitulates a key cellular effect of Cif, inhibiting the net accumulation of CFTR at the apical membrane.

Structural Analysis of aCif—aCif was identified as a putative Cif-like EH because it shares with Cif a set of active-site sequence motifs that differ from those conserved among canonical EH family members (29). To test our hypothesis directly that these motifs adopt Cif-like structures in the context of the aCif sequence, we crystallized aCif and determined its structure by x-ray diffraction analysis. Although aCif protein crystals were small and needle-like (~10–20 μm thick and 100–200 μm in length), we obtained strong diffraction to 1.95 Å resolution (Table 2). Phase information was determined by molecular replacement. Phasing quality was confirmed by composite omit maps and by clear electron density for the aCif N-terminal extension, which was not included in the search model. The refined model demonstrates excellent agreement with the experimental data, yielding a final Rwork/Rfree of 0.146/0.186.

Consistent with our hydrodynamic analysis, aCif crystallized as a dimer (Fig. 4A), with one dimer per asymmetric unit in the crystal lattice. aCif exhibits the classic α/β hydrolase-fold: a β sheet consisting of seven parallel and one antiparallel β strand sandwiched by α helices (Fig. 4A). In direct comparison, aCif and Cif also possess a high degree of structural similarity (Fig. 4B). A distance-alignment search using DALI (47) showed that the known structures closest to aCif are Cif (Z-scores ≥42),

TABLE 2

| Data collection and refinement statistics | aCif | aCif-D158S |
|------------------------------------------|------|------------|
| Wavelength (Å)                          | 1.000| 1.000      |
| Space group                             | P2₁ | P2₁        |
| Unit cell dimensions                    | a, b, c (Å) | 85.7, 42.6, 86.5 | 86.1, 42.5, 86.9 |
| α, β, γ (°)                             | 90, 98.1, 90 | 90, 98.4, 90 |
| Resolution (Å)                         | 4.22–1.95 (2.00–1.95) | 4.08–2.00 (2.05–2.00) |
| Rsym (%)                                | 9.4 (38.6) | 8.8 (31.0) |
| Rwork (%)                               | 13.7 (44.4) | 17.5 (50.4) |
| I/σI (%)                                | 14.4 (3.9) | 11.1 (3.4) |
| Completeness (%)                        | 99.8 (99.9) | 96.0 (98.3) |
| Redundancy (%)                          | 4.1 (4.1) | 2.4 (2.4) |

- ^a Values in parentheses are for data in the highest-resolution shell.
- ^b Rwork = Σi(θi − θi(calc))/Σi(θi(calc)), where θi and θi(calc) are the i-th and mean measurements of the intensity of reflection θi.
- ^c Rwork is a robust indicator of data quality, as described by Diederichs and Karplus (53).
- ^d Rwork = Σi(θi(calc) − θi(working set))/Σi(θi(working set)), h & e (working set).
- ^e Rwork = Σi(θi(calc) − θi(test set))/Σi(θi(test set)), h & e (test set).
- ^f Core/allowed/generously allowed/disallowed.
followed by members of the closely related fluoroacetate dehalogenase family (48) (Z-scores ~35).

To determine whether the aCif structure is more similar to canonical EH enzymes or to the Cif-like EH family, we focused our analysis on four distinguishing features of the Cif structure. The first and perhaps largest structural divergence demarcating our analysis on four distinguishing features of the Cif structure.

The second marked difference observed in the aCif structure was the presence of extra electron density bound within the active site (Fig. 4C). We had previously observed a water molecule coordinated by the ring-opening pair at the corresponding position in the original Cif structure (27). Unlike Cif, aCif was crystallized in the presence of 100 mM phosphate. Because the electron density is consistent with a tetrahedral geometry (Fig. 4C), it appears most likely that it represents a phosphate group occupying the substrate-binding site. To assess the ability of phosphate to act as a competitive inhibitor of aCif EH activity, we compared the specific activities of aCif for (S)-styrene oxide in buffers containing either 20 mM phosphate or 20 mM MOPS. The difference in specific activity was not significant (Fig. 3B). Therefore, we conclude that the candidate phosphate molecule observed in the structure is most likely an artifact of the crystallization conditions, rather than a physiologically relevant competitive inhibitor.
Mutation of the aCif Catalytic Nucleophile—Next, we sought to probe the catalytic activity of aCif. Based on sequence and structural comparison to Cif and other EHs, we predicted that Asp-158 serves as the catalytic nucleophile of aCif and that mutation of this residue to a Ser would ablate the EH activity. We generated this point mutant and purified aCif-D158S protein by the same method used for the WT protein. We assayed for hydrolysis of (S)-styrene oxide and observed the expected complete loss of enzyme activity (Fig. 5A).

To assess whether this loss of activity is due to localized changes in the active site or more global effects on aCif structure, we also determined the crystal structure of aCif-D158S. Refinement of the aCif crystallization conditions yielded diffraction-quality protein crystals similar in shape and size to those obtained with the WT protein. Phase information was again obtained by molecular replacement, using the aCif-WT structure as the search model. The aCif-WT and D158S models align with a root mean square deviation of 0.50 Å over 4723 atoms (Fig. 5B), confirming that the mutation does not disrupt the overall fold of the protein. The side chain of the mutant Ser at position 158 is pointed away from the active site and forms hydrogen bonds with the same backbone residues used to coordinate the Asp side chain in the WT structure (Fig. 5C). Additionally, we again found a phosphate molecule coordinated in the putative substrate binding position. We therefore conclude that Asp-158 plays an essential role in the active site, suggesting a hydrolytic mechanism shared with other EH enzymes.

Genetic Regulation of aCif—Comparison of the Acinetobacter and Pseudomonas cif operons reveals a conserved organization (Fig. 6A) that extends the structural and functional simi-

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**FIGURE 5.** Enzymatic and structural characterization of aCif-D158S. A, WT aCif and aCif-D158S were assayed for the specific activity of hydrolysis of (S)-styrene oxide using a calibrated adrenochrome assay. Values shown are mean ± S.E. (***, p < 0.001; n.s., not significant; n = 3). B, aCif (green) and aCif-D158S (orange) are shown as C traces following least-square superposition. C, the residue at nucleophile position 158 is shown for aCif (green) and aCif-D158S (orange) hydrogen bonding with Leu-159, as well as Phe-91 of the HGFG motif.

**FIGURE 6.** A, the cif operons of Pseudomonas and Acinetobacter are compared, with the genes and intergenic regions displayed to relative scale. The Acinetobacter operon does not possess a putative major facilitator superfamily (MFS) transporter gene that is found in the Pseudomonas operon. Sequence alignments were performed with ClustalW, and the percent identity was then calculated. B, qRT-PCR was performed on cultures of A. nosocomialis incubated with epoxide, or with a cifR gene deletion. Expression was not measured for cifR in the cifR deletion strain. Expression levels were compared with the values for the corresponding gene in the WT strain with buffer, using Student’s unpaired t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001; n = 3).
larieties between the Cif and aCif proteins. In \textit{P. aeruginosa}, the \textit{cifR} gene encodes a transcriptional repressor that is transcribed divergently from the \textit{cif} operon (Fig. 6A). CifR binds to the intergenic sequence between the \textit{cifR} and \textit{morB} genes and blocks transcription in both directions; repression is relieved in the presence of various epoxides (21, 46). Based on its 50% sequence identity to cifR, we hypothesized that the WP_004703794 locus encodes a functionally equivalent \textit{Acinetobacter} CifR (“aCifR”) protein that can regulate \textit{cif} expression in \textit{A. nosocomialis} by the same mechanisms observed previously in \textit{P. aeruginosa}.

To test this proposal, we first investigated the ability of aCifR to act as a transcriptional repressor by monitoring \textit{cif} expression by qRT-PCR in WT \textit{A. nosocomialis} strain RUH2624 and in a mutant strain carrying a clean deletion of the \textit{cifR} gene. Consistent with our hypothesis, relative \textit{cif} expression increased by nearly 2 orders of magnitude in the mutant lacking aCifR compared with the expression seen in WT (Fig. 6B, two leftmost columns).

Next, we tested the ability of epoxides to increase transcription of both \textit{cif} and \textit{cifR} genes, which are coordinately repressed by CifR in \textit{P. aeruginosa} (46). Gene expression was monitored by qRT-PCR in WT \textit{A. nosocomialis} cultured in the absence or presence of three epoxide compounds. One of these was epi-bromohydrin, a known activator of \textit{cif} transcription in \textit{P. aeruginosa} (46). However, because our substrate profiling indicated that aCif has a strong preference for (S)-styrene oxide (Fig. 3B), we also chose to examine both stereoisomers of styrene oxide, because the racemic mixture has previously been shown to induce Cif expression in \textit{P. aeruginosa} (21). In each case, we found the epoxide compounds were able to induce both \textit{cif} and \textit{cifR} expression in \textit{A. nosocomialis} by approximately 1 order of magnitude (Fig. 6B).

The two stereoisomers of styrene oxide yielded similar levels of induction, and both were modestly more effective than epi-bromohydrin. This suggests that all three compounds are able to interact with an effector-binding site on aCifR. The (R)- and (S)-stereoisomers of styrene oxide are likely to have similar affinities, because they should have a similar ability to cross the bacterial membrane. However, a direct comparison of potency with epibromohydrin is difficult, because it is likely to be more hydrophilic and thus may have different intracellular bioavailability. In any case, none of the candidate inducers were able to fully de-repress \textit{cif} expression to the level observed in the \textit{ΔcifR} background, suggesting that physiological compounds could achieve even higher levels of activation.

Taken together, these results suggest that the \textit{Acinetobacter} \textit{cifR} gene encodes an epoxide-sensitive repressor capable of blocking transcription of both \textit{cifR} and \textit{cif}. Upon binding to an epoxide effector, the aCifR protein would then release from its DNA binding site and allow transcription to proceed at both operons. Thus, both the genetic control and biochemical functionality of the \textit{Acinetobacter} \textit{cif} gene are closely comparable with those of its \textit{Pseudomonas} counterpart.

Concluding Remarks—Analysis of National Nosocomial Infections Surveillance (NNIS) data from 1986 to 2003 showed that \textit{A. baumannii} was associated with 5–10% of ICU pneumonia cases. It was also the only pathogen in the survey that showed a significant increase in frequency of association over time (51). Due to high levels of antibiotic resistance, recent efforts have focused on the development of drugs that target \textit{Acinetobacter} virulence factors (52). In the present study, we sought to determine whether Cif-like sequence motifs can correctly identify homologous epoxide hydrolases in opportunistic pathogens other than \textit{P. aeruginosa}. Indeed, our evidence overwhelmingly suggests that \textit{A. nosocomialis} and \textit{A. baumannii} strains possess systems similar to Cif, consisting of a secreted EH enzyme that is genetically regulated by an epoxide-sensitive TetR family repressor. These systems likely enable the bacteria to reduce the abundance of CFTR in polarized human airway epithelial cells, and thus to inhibit mucociliary clearance during the establishment of airway infections. As a result, the aCif protein may represent a new therapeutic target, either alone or as part of a broader strategy to combat this emerging threat to public health.

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REFERENCES
1. Riordan, J. R. (2008) CFTR function and prospects for therapy. \textit{Annu. Rev. Biochem.} 77, 701–726
2. Davies, J. C., and Bilton, D. (2009) Bugs, biofilms, and resistance in cystic fibrosis. \textit{Respir. Care} 54, 628–640
3. Cantin, A. M., Hanrahan, J. W., Bilodeau, G., Ellis, L., Dupuis, A., Liao, J., Zielenski, J., and Durie, P. (2006) Cystic fibrosis transmembrane conductance regulator function is suppressed in cigarette smokers. \textit{Am. J. Respir. Crit. Care Med.} 173, 1139–1144
4. Clunes, L. A., Davies, C. M., Cookley, R. D., Aleksandrov, A. A., Henderson, A. G., Zeman, K. L., Worthington, E. N., Gentzsch, M., Kreda, S. M., Cholon, D., Bennett, W. D., Riordan, J. R., Boucher, R. C., and Tarran, R. (2012) Cigarette smoke exposure induces CFTR internalization and insolubility, leading to airway surface liquid dehydration. \textit{FASEB J.} 26, 533–545
5. Kreindler, J. L., Jackson, A. D., Kemp, P. A., Bridges, R. J., and Danahay, H. (2005) Inhibition of chloride secretion in human bronchial epithelial cells by cigarette smoke extract. \textit{Am. J. Physiol. Lung Cell. Mol. Physiol.} 288, L894–L902
6. Sloane, P. A., Shastery, S., Wilhelm, A., Courville, C., Tang, L. P., Backer, K., Levin, E., Raju, S. V., Li, Y., Mazur, M., Byan–Parker, S., Grizzle, W., Sorscher, E. J., Dransfield, M. T., and Rowe, S. M. (2012) A pharmacologic approach to acquired cystic fibrosis transmembrane conductance regulator dysfunction in smoking related lung disease. \textit{PLoS ONE} 7, e39809
7. Kochanek, K. D., Xu, J., Murphy, S. L., Minino, A. M., and Kung, H.-C. (2011) Deaths. Final Data for 2009. \textit{Natl. Vital Stat. Rep.} 60, 1–117
8. Erb-Downward, J. R., Thompson, D. L., Han, M. K., Freeman, C. M., McCluskey, L., Schmidt, L. A., Young, V. B., Toews, G. B., Curtis, J. L., Sundaram, B., Martinez, F. J., and Huffnagle, G. B. (2011) Analysis of the lung microbiome in the “healthy” smoker and in COPD. \textit{PLoS ONE} 6, e16384
9. Montero, M., Domínguez, M., Orozco-Levi, M., Salvador, M., and Nobel, H. (2009) Mortality of COPD patients infected with multi-resistant \textit{Pseudomonas aeruginosa}. A case and control study. \textit{Infection} 37, 16–19
10. Murphy, T. F. (2009) \textit{Pseudomonas aeruginosa} in adults with chronic obstructive pulmonary disease. \textit{Curr. Opin. Pulm. Med.} 15, 138–142
11. Parameswaran, G. I., and Sethi, S. (2012) \textit{Pseudomonas} infection in
chronic obstructive pulmonary disease. *Future Microbiol.* 7, 1129–1132
12. Koulenti, D., and Rello, J. (2006) Gram-negative bacterial pneumonia. *Aetiology and management. Carr. Opin. Pulm. Med.* 12, 198–204
13. Visca, P., Seifert, H., and Towner, K. J. (2011) *Acinetobacter* infection. An emerging threat to human health. *ILRBM Life Sci.* 63, 1048–1054
14. Rice, L. B. (2008) Federal funding for the study of antimicrobial resistance in nosocomial pathogens. No ESKAPE. *J. Infect. Dis.* 197, 1079–1081
15. Howard, A., O’Donoghue, M., Feeoney, A., and Sleator, R. D. (2012) *Acinetobacter baumannii*. An emerging opportunistic pathogen. *Virulence* 3, 243–250
16. Haibly, N., Ciofu, O., and Bjarnholt, T. (2010) *Pseudomonas aeruginosa* biofilms in cystic fibrosis. *Future Microbiol.* 5, 1663–1674
17. Dieme, S. M., and Rolain, J. M. (2013) Investigation of antibiotic resistance in the genomic era of multidrug-resistant Gram-negative bacilli, especially *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter*. *Expert Rev. Anti-Infect. Ther.* 11, 277–296
18. Gregurich, P. E., Hudcova, J., Lei, Y., Sarwar, A., and Craven, D. E. (2012) Management and prevention of ventilator-associated pneumonia caused by multidrug-resistant pathogens. *Expert Rev. Respir. Med.* 6, 533–555
19. Horsley, A., and Jones, A. M. (2012) *Pseudomonas aeruginosa* secreted protein PA2934 decreases apical membrane expression of the cystic fibrosis transmembrane conductance regulator. *Infect. Immun.* 75, 3902–3912
20. MacEachran, D. P., Ye, S., Bomberger, J. M., Hogan, D. A., and O’Toole, G. A. (2012) Epoxide-mediated CifR repression of *cif* gene expression utilizes two binding sites in *Pseudomonas aeruginosa*. *J. Bacteriol.* 194, 5315–5324
21. Swietacka-Urban, A., Moreau-Marquis, S., MacEachran, D. P., Connolly, J. P., Stanton, C. R., Su, J. R., Barnaby, R., O’Toole, G. A., and Stanton, B. A. (2006) *Pseudomonas aeruginosa* inhibits endocytic recycling of CFTR in polarized human airway epithelial cells. *Ann. J. Physiol. Cell Physiol.* 290, C862–C872
22. Bomberger, J. M., MacEachran, D. P., Coutermarsh, B. A., Ye, S., O’Toole, G. A., and Stanton, B. A. (2009) Long-distance delivery of bacterial virulence factors by *Pseudomonas aeruginosa* outer membrane vesicles. *PLoS Pathog.* 5, e1000382
23. Wilson, R. T., Roberts, P., Munro, N., Rutman, A., Hinsa, S. M., Toutain, C. M., and O’Toole, G. A. (2005) Epoxide hydrolase virulence factor. *J. Clin. Invest.* 79, 221–229
24. Wilson, R. T., Roberts, P., and Cole, P. (1985) Effect of bacterial products on human ciliary function in vitro. *Thorax* 40, 125–131
25. Morisseau, C., Bomberger, J. M., Stanton, B. A., Hammock, B. D., O’Toole, G. A., and Madden, D. R. (2010) Crystal structure of the CifR inhibitory factor Cif reveals novel active-site features of an epoxide hydrolase virulence factor. *J. Bacteriol.* 192, 1785–1795
26. Bahl, C. D., and Madden, D. R. (2012) *Pseudomonas aeruginosa* Cif defines a distinct class of α/β epoxide hydrolases utilizing a His/Tyr ring-opening pair. *Protein Pept. Lett.* 19, 186–193
27. Nemeč, A., Krizova, L., Maixnerova, M., van der Reijden, T. J., Deschaght, P., Passet, V., Vaneechouette, M., Brise, S., and Drijksoort, L. (2011) Genotypic and phenotypic characterization of the *Acinetobacter calcoaceti cus-Acinetobacter baumannii* complex with the proposal of *Acinetobacter pittii* sp. nov. (formerly *Acinetobacter* genomic species 3) and *Acinetobacter nosocomialis* sp. nov. (formerly *Acinetobacter* genomic species 13TU). *Res. Microbiol.* 162, 393–404
28. Shanks, R. M., Caiazza, N. C., Hinsa, S. M., Toutain, C. M., and O’Toole, G. A. (2006) *Saccharomyces cerevisiae*-based molecular tool kit for manipulation of genes from Gram-negative bacteria. *Appl. Environ. Microbiol.* 72, 5027–5036
29. Hoeijmakers, R., and DeRosa, J. (2008) Purification, crystallization and preliminary x-ray diffraction analysis of Cif, a virulence factor secreted by *Pseudomonas aeruginosa*. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 66, 26–28
30. Luft, J. R., Collins, R. J., Fehrman, N. A., Lauricella, A. M., Veatch, C. K., and DeTitta, G. T. (2003) A deliberate approach to screening for initial crystallization conditions of biological macromolecules. *J. Struct. Biol.* 142, 170–179
31. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX. A comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr.* D 66, 213–221
32. Adams, P. D., Grosse-Kunstleve, R. W., Hung, L. W., Iorger, T. R., McCoy, A. J., Moriarty, N. W., Read, R. J., Sacchettini, J. C., Sauter, N. K., and Terwilliger, T. C. (2002) PHENIX. Building new software for automated crystallographic structure determination. *Acta Crystallogr.* 58, 1948–1954
33. Bahl, C. D., and Madden, D. R. (2010) Epoxide-mediated CifR repression of *cif* gene expression utilizes two binding sites in *Pseudomonas aeruginosa*. *J. Bacteriol.* 192, 2271–2277
34. Wilson, R., Pitt, T., Taylor, G., Watson, D., MacDermot, J., Sykes, D., Roberts, D., and Cole, P. (1987) Pyocyanin and 1-hydroxyphenazin produced by *Pseudomonas aeruginosa* inhibit the beating of human respiratory cilia in vitro. *J. Clin. Invest.* 79, 221–229
35. Wilson, R., Roberts, D., and Cole, P. (1985) Effect of bacterial products on human ciliary function in vitro. *Thorax* 40, 125–131
36. Bahl, C. D., and Madden, D. R. (2012) *Pseudomonas aeruginosa* Cif defines a distinct class of α/β epoxide hydrolases utilizing a His/Tyr ring-opening pair. *Protein Pept. Lett.* 19, 186–193
37. Nemec, A., Krizova, L., Maixnerova, M., van der Reijden, T. J., Deschaght, P., Passet, V., Vaneechouette, M., Brise, S., and Drijksoort, L. (2011) Genotypic and phenotypic characterization of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex with the proposal of *Acinetobacter pittii* sp. nov. (formerly *Acinetobacter* genomic species 3) and *Acinetobacter nosocomialis* sp. nov. (formerly *Acinetobacter* genomic species 13TU). *Res. Microbiol.* 162, 393–404
D. W., and Hammock, B. D. (2000) Biochemical evidence for the involvement of tyrosine in epoxide activation during the catalytic cycle of epoxide hydrolase. J. Biol. Chem. 275, 23082–23088

50. Arand, M., Cronin, A., Adamska, M., and Oesch, F. (2005) Epoxide hydrolases. Structure, function, mechanism, and assay. Methods Enzymol. 400, 569–588

51. Gaynes, R., and Edwards, J. R. (2005) Overview of nosocomial infections caused by Gram-negative bacilli. Clin. Infect. Dis. 41, 848–854

52. López-Rojas, R., Smani, Y., and Pachón, J. (2013) Treating multidrug-resistant Acinetobacter baumannii infection by blocking its virulence factors. Expert Rev. Anti Infect. Ther. 11, 231–233

53. Diederichs, K., and Karplus, P. A. (1997) Improved R-factors for diffraction data analysis in macromolecular crystallography. Nat. Struct. Biol. 4, 269–275