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Cif Is Negatively Regulated by the TetR Family Repressor CifR[∇]

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We previously reported that the novel *Pseudomonas aeruginosa* toxin Cif is capable of decreasing apical membrane expression of the cystic fibrosis transmembrane conductance regulator (CFTR). We further demonstrated that Cif is capable of degrading the synthetic epoxide hydrolase (EH) substrate *S*-NEPC [(2*S*,3*S*)-*trans*-3-phenyl-2-oxiranylmethyl 4-nitrophenol carbonate], suggesting that Cif may be reducing apical membrane expression of CFTR via its EH activity. Here we report that Cif is capable of degrading the xenobiotic epoxide epibromohydrin (EBH) to its vicinal diol 3-bromo-1,2-propanediol. We also demonstrate that this epoxide is a potent inducer of *cif* gene expression. We show that the predicted TetR family transcriptional repressor encoded by the PA2931 gene, which is immediately adjacent to and divergently transcribed from the *cif*-containing, three-gene operon, negatively regulates *cif* gene expression by binding to the promoter region immediately upstream of the *cif*-containing operon. Furthermore, this protein-DNA interaction is disrupted by the epoxide EBH *in vitro*, suggesting that the binding of EBH by the PA2931 protein product drives the disassociation from its DNA-binding site. Given its role as a repressor of *cif* gene expression, we have renamed PA2931 as CifR. Finally, we demonstrate that *P. aeruginosa* strains isolated from cystic fibrosis patient sputum with increased *cif* gene expression are impaired for the expression of the *cifR* gene.

Pseudomonas aeruginosa is a ubiquitous opportunistic pathogen known to infect a variety of organisms, including plants, nematodes, fruit flies, and humans (19, 30, 49). Disease in humans shows a relative lack of tropism, as infections caused by *P. aeruginosa* can be found in tissues as varied as the skin, ocular epithelia, and the lung (3, 28, 31). This pathogenic plasticity is largely due to the presence of a multiplicity of virulence factors which allow for colonization and the establishment of infection (1). This arsenal of virulence factors includes the well-characterized type three secretion system and its effector molecules, elastases, phospholipases, phenazines, and rhamnolipids (6, 9, 25, 27, 44, 47, 52). These virulence factors contribute to both acute and chronic infections associated with burn wounds, ocular infections, *P. aeruginosa* ventilator-associated pneumonia, and cystic fibrosis (CF).

The genetic disease CF is the result of heritable mutations within the cystic fibrosis transmembrane conductance regulator (CFTR), the most common of which is the deletion of the phenylalanine at position 508 (18, 37, 38, 48). In healthy airway epithelia cells, CFTR acts to directly regulate the flux of Cl⁻ ions and, indirectly, the flux of Na⁺ ions and water across the apical membrane (22, 37). Airway epithelial cells in CF patients demonstrate decreased functional CFTR at their apical membrane; thus, there is altered Cl⁻ and Na⁺ ion and water flux across this membrane, resulting in increased viscosity and decreased height of the periciliary fluid, which in turn leads to decreased ciliary beating and a subsequent loss of the mucociliary elevator, a key component of the innate immune system. This breach in mucociliary clearance allows for a number of different pathogens, including *P. aeruginosa*, to colonize the CF

lung and establish chronic infections (5). Following initial colonization by environmental isolates, these bacteria undergo a series of phenotypic changes including the acquisition of auxotrophy for various nutrients, alterations in quorum-sensing machinery, and the overproduction of the exopolysaccharide alginate (11–14, 41). The overproduction of alginate results in the mucoid phenotype associated with *P. aeruginosa* sputum isolates from chronically infected CF patients. The mucoid phenotype has been well studied and is believed to be the result of selective pressures including exposure to antibiotics as well as the host innate immune system (12, 13, 35).

We previously described a novel *P. aeruginosa* toxin that is packaged into outer membrane vesicles and is capable of dramatically reducing apical membrane expression of CFTR in several different epithelial cell lines (29, 46). This toxin, known as Cif, was predicted to belong to the family of epoxide hydrolases (EHs) and was experimentally shown to degrade the synthetic EH substrate *S*-NEPC [(2*S*,3*S*)-*trans*-3-phenyl-2-oxiranylmethyl 4-nitrophenol carbonate]. While it is not entirely understood how this EH activity may regulate apical membrane expression of CFTR, epoxides and their metabolites have been shown to act as signaling molecules in both endothelial and epithelial cells, regulating processes as varied as vasodilation and Cl⁻ ion transport (33, 42). We found that a subset of *P. aeruginosa* strains isolated from the CF airway demonstrated a marked increase in *cif* transcription relative to laboratory strains, suggesting a role for Cif in the pathophysiology of CF.

Our previous demonstration that *cif* was differentially expressed in mucoid versus nonmucoid CF clinical isolates (29) led us to investigate the regulation of *cif* gene expression. Here we show that the Cif protein is capable of degrading the epoxide epibromohydrin (EBH) and that this xenobiotic compound induces *cif* gene expression via its ability to alter DNA binding of the TetR family regulator CifR. Finally, we show that reduced *cifR* gene expression can account for the high

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TABLE 1. Strains, plasmids, and primers used

Strain, plasmid, or primer	Relevant characteristic	Reference or source
Strain		
<i>S. cerevisiae</i> INVSc1	<i>MATa his3D1 leu2 trp1-289 ura3-52 MAT his3D1 leu2 trp1-289 ura3-52</i>	Invitrogen
<i>E. coli</i> Top10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) f80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 araΔ139</i> Δ(<i>ara leu</i>)7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i>	Invitrogen
SMC3505	<i>E. coli</i> Top10 + pDPM73 (Cif-His)	29
SMC3814	<i>E. coli</i> Top10 + pDPM79 (CifR-His)	This study
<i>P. aeruginosa</i> PA14	Wild type	
SMC1584	<i>P. aeruginosa</i> clinical isolate	29
SMC1586	<i>P. aeruginosa</i> clinical isolate	29
SMC1591	<i>P. aeruginosa</i> clinical isolate	29
SMC1593	<i>P. aeruginosa</i> clinical isolate	29
SMC3816	PA14 ΔPA2931 + pDPM79	This study
SMC3817	SMC1584 + pMQ71	This study
SMC3818	SMC1584 + pDPM79	This study
SMC3819	SMC1586 + pMQ71	This study
SMC3820	SMC1586 + pDPM79	This study
Plasmid		
pMQ71	Arabinose-inducible expression vector Cb ^r Ap ^r URA3	40
pMQ30	Allelic replacement vector; Gm ^r	40
pDPM79	PA2931 hexa-histidine fusion in pMQ71; Ap ^r Cb ^r URA3	This study
pDPM84	PA2931 in-frame deletion plasmid; Gm ^r	This study
Primer		
PA2934_rt_1	5'-CTCCTGGCCGCGCATCGCCCTGACCTTCTCC-3'	
PA2934_rt_2	5'-CCATTCGTACCAGGTCTGGCCGAAGCCGTGC-3'	
RplU_rt_1	5'-GGTGGCAAGCAGCACAAAGTCACCG-3'	
RplU_rt_2	5'-GCGGACCTTGTCTGACGCGCCGTGG-3'	
PA2931_rt_for	5'-CCAGGGCTTCGCGGAACAGCCCTTCC-3'	
PA2931_rt_rev	5'-GACACCGCCCTGCAGCGAGCCATGG-3'	
Cif_northern_for	5'-BT-GCCGCGGCCTCCTGGCCGGCATCGCCC-3' ^a	
Cif_northern_rev	5'-BT-CGCGCACCGTTTCGCCGCTATAGCCGG-3'	
PA2931_ko_1	5'-CGCCAGGGTTTTCCAGTCACGACGTTGTA AAAACGACG GCTGGAAGGCTCCAAG GTAGAATGGATGG-3'	
PA2931_ko_2	5'-CAAGTAAATTTATAGTGATCGATACAAATATGAGCG GGTCGCATCAGGCCGCC-3'	
PA2931_ko_3	5'-GGCGGCCTGATGCGACCCGCTCATATTTGTATC GATCACTATAAAATTTACTTG-3'	
PA2931_ko_4	5'-GTGAGCGGATAACAATTTACACAGGAAACAGCTAT GACCGCAGGCGCTCGCGG AAGCCTTGC-3'	
PA2931_His_for	5'-GAAAATCTTCTCTCATCCGCTCAATGATGATGATG ATGATGGGGCCAGGCGCG AGCGCCGTTTCG-3'	
PA2931_His_rev	5'-CTCCATACCCGTTTTTTTTGGGCTAGCCCAAGGA AGCACACATATGGCAACGCGAG GCAGGCCAC-3'	
EMSA_5'bt_for	5'-BT-CCTGACCTCCATTATTTGTATCGATCAC-3'	
EMSA_5'bt_rev	5'-BT-GAGCAGGTTGGACATGGTCTTTTCC-3'	
EMSA_for	5'-CCTGACCTCCATTATTTGTATCGATCAC-3'	
EMSA_rev	5'-GAGCAGGTTGGACATGGTCTTTTCC-3'	

^a BT, biotinylated.

level of *cif* gene expression observed in the nonmucoid *P. aeruginosa* CF sputum isolates.

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) unless otherwise noted (2). Growth media were supplemented with antibiotics at the following concentrations: gentamicin, 10 μg/ml (*Escherichia coli*) and 100 μg/ml (*P. aeruginosa*); and ampicillin, 150 μg/ml (*E. coli*) and 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) media at 30°C. All restriction enzymes were purchased from New England Biolabs (Beverly, MA). All plasmids were constructed in *E. coli* Top10 cells using standard protocols or in *Saccharomyces cerevisiae* INVSc1 (Invitrogen, Carlsbad, CA) using in vivo recombination and electroporated or conjugated into *P. aeruginosa* strain PA14, as previously reported (8, 40).

EBH degradation assays. EBH degradation by Cif was performed as previously described (7). Briefly, 50 μg of purified Cif was incubated with EBH at a final concentration of 10 mM in 20 mM HEPES buffer (pH 7.4) and 500 mM NaCl for 30 min at 37°C. Sodium periodate in acetonitrile was added at final concentrations of 1.67 mM and 30%, respectively, and incubated at room temperature for 1 h. Epinephrine-HCl was then added to a final concentration of 1.5 mM. The reaction was allowed to continue for 15 min at room temperature prior to removal of the supernatant and subsequent detection at a wavelength of 490 nM with a Molecular Devices SpectraMax M2 plate reader. Degradation of EBH was demonstrated by a reduction in absorbance at 490 nM.

EBH induction assays. Wild-type *P. aeruginosa* PA14 was grown overnight in LB at 37°C and subsequently diluted 1:100 in LB supplemented with EBH at the concentrations indicated for each experiment. Cultures were grown at 37°C with shaking to an optical density at 600 nm (OD₆₀₀) of 1.0. Cultures (500 μl) were centrifuged and pellets were frozen at -80°C. Samples for protein analysis were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer as previously described and subsequently resolved using SDS-PAGE. Cif protein expression was analyzed using standard Western blot-

ting as previously described (29). RNA for semiquantitative reverse transcription-PCR (sqRT-PCR) was harvested as described below.

Detection of the *morB*-PA2933-*cif* transcript. RNA for Northern blot analysis was harvested as described below. Samples were prepared, electrophoresed, and transferred as previously described (17). Briefly, 20 μ g of RNA was denatured using formaldehyde and formamide in MOPS (morpholinepropanesulfonic acid) buffer at 65°C for 15 min. Gel loading buffer was added to the denatured RNA, which was subsequently electrophoresed in a MOPS-formaldehyde-agarose gel for 1 h at 80 V. RNA was transferred to a Biotodyne B modified nylon membrane (Thermo Scientific, Rockford, IL) using a standard wicking transfer for 4 to 8 h. Following cross-linking and blocking, 500 ng of a biotinylated *cif*-specific probe was added to the blocking buffer and incubated at 65°C for 12 h. The *cif* probe was synthesized using the biotinylated primers Cif_northern_for and Cif_northern_rev (Table 1) in a standard PCR followed by gel purification. Membranes were washed with SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and SDS buffer and subsequently labeled with a streptavidin-horseradish peroxidase conjugate followed by detection using a LightShift chemiluminescence kit per the manufacturer's instructions (Pierce, Rockford, IL).

Plasmid construction. Construction of the PA2931 deletion plasmid was performed as previously described (40) using primers PA2931_ko_1, PA2931_ko_2, PA2931_ko_3, and PA2931_ko_4. Amplicons were created using primer pair ko_1 and ko_2 and primer pair ko_3 and ko_4 and were recombined using *S. cerevisiae* INVSc1 (Invitrogen) into plasmid pMQ30 linearized with restriction enzymes EcoRI, HindIII, and BamHI. The resulting recombinants were lysed and plasmids recovered by using standard techniques. Plasmid pDPM84 was transformed into wild-type *P. aeruginosa* strain PA14 as previously described (8), and transformants were selected for on solid LB media supplemented with gentamicin. Merodiploids were resolved on solid LB media supplemented with 10% sucrose as previously described (45).

Plasmid pDPM79 containing the C-terminal, six-histidine-tagged variant of PA2931 was constructed using standard yeast recombination techniques. Briefly, the PA2931 open reading frame (ORF) was amplified using primers PA2931_His_for and PA2931_His_rev, which contain regions homologous to the PA2931 ORF and the plasmid pMQ71. The resulting amplicon was recombined into the plasmid pMQ71.

Purification of the Cif protein. Purification of the histidine-tagged Cif protein was performed as previously described (29).

Purification of the CifR protein. The hexa-histidine-tagged variant of the PA2931 protein was expressed from the arabinose-inducible pDPM79 plasmid in *E. coli* Top10 cells (Invitrogen, Carlsbad, CA). Cells containing the plasmid were grown overnight at 37°C in LB supplemented with ampicillin, diluted 1:100 in 1 liter of LB supplemented with ampicillin and 0.2% arabinose, and incubated with shaking at 37°C for 8 h. Cultures were centrifuged at 7,000 \times g for 20 min. Cell pellets were then mechanically lysed via French pressure lysis. Lysates were centrifuged at 20,000 \times g for 30 min to remove cellular debris. Supernatants from centrifuged lysates were 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 PA2931 protein eluting at \sim 100 mM. Fractions containing PA2931 were pooled and concentrated using Amicon Ultra-15 10-kDa centrifugation columns (Millipore, Billerica, MA). The protein solution was then dialyzed against 20 mM Tris-500 mM NaCl, pH 7.5. The protein concentration was determined by utilizing a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Protein purity was determined using SDS-PAGE followed by staining with Coomassie blue.

EMSA. Electromobility shift assays (EMSA) were performed using a Light-Shift chemiluminescence EMSA kit (Pierce, Rockford, IL) per the manufacturer's instructions. The PA2931-*morB* intergenic region was amplified using primers EMSA_5'bt_for, EMSA_5'bt_rev, EMSA_for, and EMSA_rev, resulting in either the biotinylated probe or the cold-competitor DNA. The biotinylated probe was then incubated with the purified PA2931 protein for 30 min. Samples were loaded onto 10 \times Tris-borate-EDTA-PAGE gels and electrophoresed. Following electrophoresis, samples were electro-transferred to a Biotodyne nylon membrane (Rockford, IL) in 0.5 Tris-borate-EDTA. Samples were then UV cross-linked to the membrane and blocked according to the manufacturer's instructions. Labeling and subsequent detection were carried out using a streptavidin-horseradish peroxidase conjugate and chemiluminescence, as described by the manufacturer. When EBH was included in the EMSA experiments, it was added simultaneously with the DNA probe and protein prior to incubation at 37°C.

RNA purification and cDNA synthesis. RNA purification and cDNA synthesis were performed as previously described (29) with the following modifications: strains for sqRT-PCR were grown overnight in LB medium and subsequently diluted 1:100 in LB medium and grown to an OD₆₀₀ of 1.0. Cultures (500 μ l)

were harvested and centrifuged at 16,000 \times g for 2 min, and the cell pellets were frozen at -80°C . Strains were grown in triplicate, and two samples were harvested per replicate.

RESULTS

Cif degrades the epoxide EBH. We previously reported that the *P. aeruginosa* secreted toxin Cif is capable of degrading the generic EH substrate *S*-NEPC, suggesting that this protein may act as an EH (29). A previous report by Jacobs et al. identified a soil pseudomonad capable of surviving on and degrading the xenobiotic epoxide EBH (24). Further analysis demonstrated that the biochemical activity produced by this pseudomonad was the result of a secreted protein with a mass similar to that of the Cif protein. Functional analysis of the secreted protein demonstrated that it was an EH. The amino acid sequence of the N terminus of this EH and three CNBr-generated fragments of the protein shared up to 40% identity with the Cif protein. The work by Jacobs and colleagues, as well as our previous demonstration that Cif could degrade *S*-NEPC, led us to hypothesize that Cif may be capable of degrading the xenobiotic epoxide EBH.

To assay EH activity with EBH as the substrate, we monitored the production of the vicinal diol of EBH, 3-bromo-1,2-propanediol. This product is readily oxidized by periodate, producing aldehyde and ketone variants of 3-bromo-1,2-propanediol. The oxidation of 3-bromo-1,2-propanediol by periodate results in the reduction of periodate. The reduction of 3-bromo-1,2-propanediol by periodate can be detected using epinephrine, which itself is readily oxidized by periodate, resulting in a red color. The reduction of periodate results in an inability to oxidize epinephrine and hence no colorimetric change (7). Thus, the degradation of EBH by Cif can be readily assayed based on the fact that as more EBH is degraded, more periodate is reduced, and hence less epinephrine is oxidized (Fig. 1A).

When we incubated EBH with the buffer alone, we observed a red color detected at OD₄₉₀, indicating oxidation of the epinephrine due to the presence of periodate (data not shown). However, when purified Cif was incubated with EBH, we observed an absorbance threefold less than what we observed in the no-protein control, consistent with our hypothesis that Cif is an EH and is capable of degrading EBH (Fig. 1B). Furthermore, when Cif was heated to 95°C for 20 min (heat kill), thus denaturing the protein, we saw a complete loss of the EH activity compared to activity with the non-heat-treated protein. As an additional control, supernatant from *E. coli* Top10 cells harboring the empty vector pMQ70 was fractionated using conditions similar to those used to purify the Cif protein. Neither the supernatant alone nor fractions derived from the supernatant of the empty vector strain demonstrated any degradation of EBH (data not shown).

To further test Cif's ability to degrade EBH, we performed a dose-response experiment, incubating EBH with decreasing amounts of purified Cif (Fig. 1C). Consistent with Cif's ability to degrade EBH, we observed decreasing EBH degradation with decreasing concentrations of the Cif protein.

Both *cif* gene and Cif protein expression are induced by EBH. The expression of a variety of catabolic genes is induced in the presence of their cognate substrates. Typically, this in-

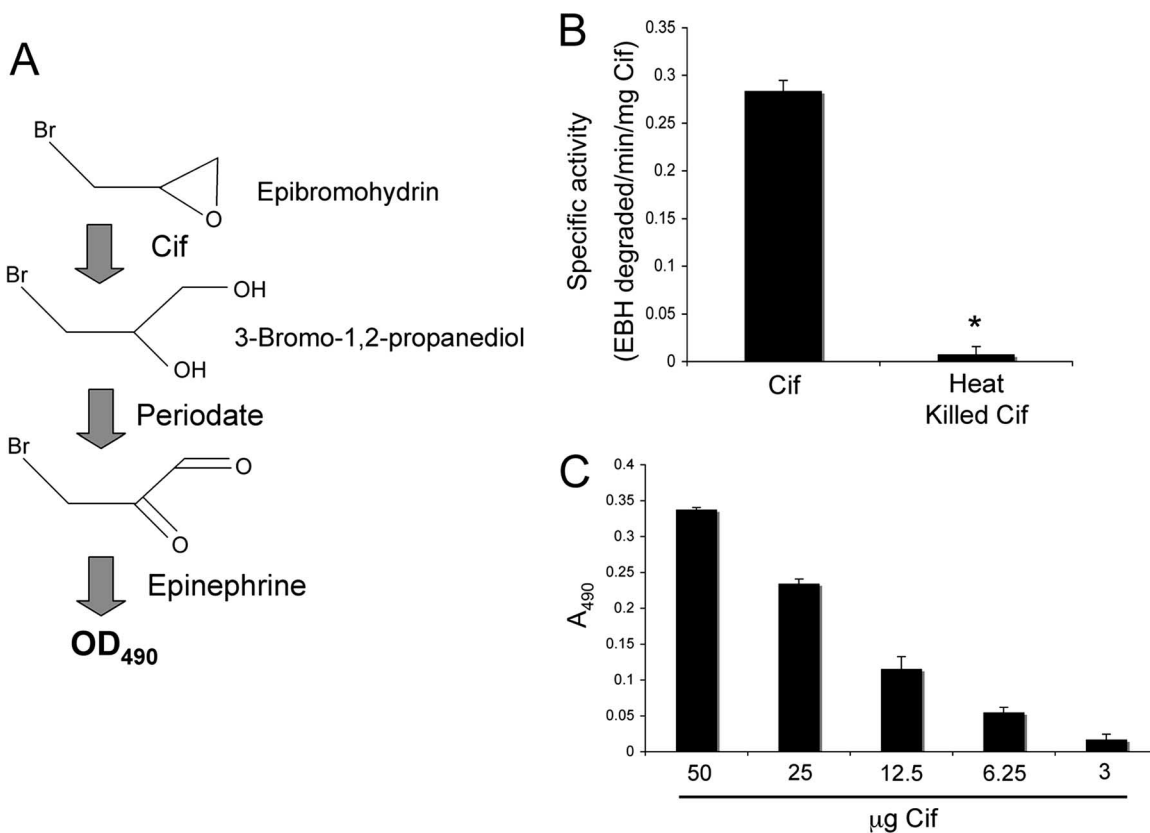


FIG. 1. Cif degrades the epoxide EBH. The degradation of epoxides by EHs creates highly reactive vicinal diols. The colorimetric detection of these diols is outlined in panel A. Generation of the vicinal diol of EBH by Cif can be detected spectrophotometrically through the oxidation of 3-bromo-1,2-propanediol by periodate and subsequent analysis of the redox state of epinephrine added to the reaction. (B) Purified Cif (50 μg) was incubated with 10 mM EBH, and absorbance at 490 nm was monitored. Also shown is a heat-inactivated protein (heat kill) control. Data were normalized to samples containing buffer alone. *, P value of <0.05 . Values are presented as specific activity (EBH degraded/min/mg Cif). (C) Dose response of EBH degradation by Cif. Bar 1 shows 10 mM EBH and 50 μg Cif; bar 2, EBH and 25 μg Cif; bar 3, EBH and 12.5 μg Cif; bar 4, EBH and 6.25 μg Cif; and bar 6, EBH and 3 μg Cif. Samples were incubated at 37°C for 15 min. Values are presented as the OD_{490} , with the OD_{490} measurements of each condition subtracted from the control condition containing EBH alone.

creased gene expression is the result of a regulatory protein that either induces gene expression or derepresses expression in the presence of the cognate effector molecule. The observation that Cif is capable of degrading the epoxide EBH led us to hypothesize that *cif* gene expression may be positively regulated in the presence of this molecule.

To assess the response of *cif* gene expression to EBH, we grew wild-type *P. aeruginosa* PA14 in the presence of increasing concentrations of EBH and then performed sqRT-PCR. We found that the addition of EBH to *P. aeruginosa* cultures resulted in a dose-dependent increase in *cif* transcript levels, while the transcript levels of the control *rplU* transcript remained largely unchanged (Fig. 2A).

To further confirm these results, wild-type *P. aeruginosa* cultures grown under identical conditions were assayed by Western blotting (Fig. 2B). We also found a dose-dependent increase in Cif protein levels. These results demonstrate that EBH is a potent inducer of *cif* gene expression, resulting in increased Cif protein levels.

***cif* is transcribed as part of a three-gene operon.** The observation that EBH induced expression of the *cif* gene led us to further investigate the regulation of this gene. The chromo-

somal organization of the *cif* gene predicts that it belongs to a three-gene operon with the *morB* and PA2933 genes (Fig. 3A). To determine if in fact *cif* was transcribed as part of a polycistronic mRNA, we performed Northern analysis using a biotinylated probe specific for the *cif* gene. RNA was isolated from wild-type PA14, the Δcif mutant, and the ΔPA2931 mutant and probed for *cif* gene expression.

A single band was detected at 4.2 kb, consistent with the prediction that *cif* is cotranscribed with the *morB* and PA2933 genes (Fig. 3B). Due to relatively low *cif* transcription levels, we were unable to detect the *cif*-containing operon transcript via Northern analysis in the wild-type genetic background. However, in a *P. aeruginosa* PA14 mutant that overexpresses *cif* as a result of a mutation in the PA2931 ORF (see below), we were able to detect the transcript of the appropriate size. These data suggest that all three genes are transcribed from the predicted promoter region immediately adjacent to the *morB* gene.

PA2931 negatively regulates *cif* gene expression. With the demonstration that *cif* gene expression was variable and inducible, we next sought to identify genes that regulate *cif* gene expression. Divergently transcribed from the *morB* gene is the

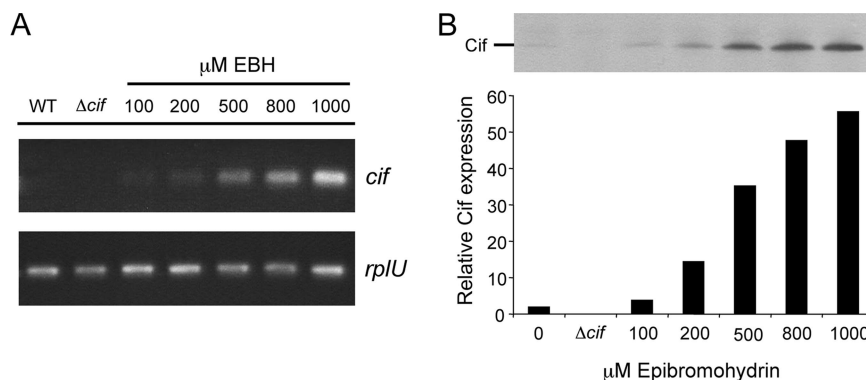


FIG. 2. EBH induction of *cif* gene and Cif protein expression. Wild-type (WT) *P. aeruginosa* strain PA14 was grown in LB supplemented with increasing concentrations of the epoxide EBH. Expression of the *cif* gene and Cif protein in response to EBH was assayed using either sqRT-PCR (A) or Western blotting (B), respectively. The expression of *rplU*, a gene previously shown to be constitutively expressed under most laboratory conditions, served as a control (panel A). Samples for Western blotting were normalized to total protein content (panel B). The graphical interpretation of the Western blot data shown in panel B is the relative Cif expression normalized to the total protein electrophoresed.

PA2931 gene, predicted to encode a TetR family DNA-binding protein. Members of this family are known to regulate gene expression by binding to the promoter region and occluding the RNA polymerase-binding site, thus inhibiting gene expression. Via *in silico* analysis, we found a predicted promoter region immediately adjacent to the *morB*-PA2933-*cif* operon. The relative proximity and orientation of the PA2931 gene led us to hypothesize that it negatively regulates *cif* gene expression.

To test this hypothesis, a clean deletion of the PA2931 gene was created in *P. aeruginosa* PA14. A hexa-histidine-tagged variant of the cloned PA2931 gene was created in the multi-copy, arabinose-inducible plasmid pMQ71 (40) (designated pDPM79) to be used both in complementation studies here and for expression of the PA2931 protein for purification described below. RNA from these strains was isolated, and *cif* and *rplU* gene expression were assayed by using sqRT-PCR. These strains were also assayed for Cif protein expression by using Western blotting.

Deletion of the PA2931 gene resulted in a marked increase in *cif* gene expression (Fig. 3B and 4A). Introduction of the PA2931 histidine-tagged variant expressed from the plasmid pDPM79 into the PA2931 deletion strain resulted in a restoration of wild-type levels of *cif* transcript.

The marked increase in expression seen in the ΔPA2931 strain, compared to that in the wild-type strain, required the use of a low number of PCR cycles in order to demonstrate the difference in expression and to capture *cif* expression in the ΔPA2931 strain during the linear range of the PCR. These PCR conditions resulted in almost undetectable levels of the *cif* amplicon in the wild-type strain. The use of a higher number of cycles on identical samples demonstrated that the *cif* transcript was detectable in the wild-type and complemented strains but not in the Δ*cif* mutant strain, as would be expected (data not shown).

These data were further confirmed by using Western blotting. Cif protein expression was increased in the PA2931 deletion strain carrying the empty vector pMQ71, while strains expressing the histidine-tagged variant of the PA2931 protein from the plasmid pDPM79 demonstrated reduced Cif protein expression compared to that of the vector control (Fig. 4B).

These data taken together demonstrate that the protein encoded by the PA2931 gene negatively regulates *cif* gene transcription, resulting in decreased Cif protein expression. Based on these data, we have renamed the PA2931 gene *cifR*, reflecting its ability to repress *cif* gene expression.

CifR shows EBH-dependent binding upstream of the *morB*-PA2933-*cif* operon. The TetR family of repressors has previously been shown to bind directly to the promoter region upstream of the genes they regulate in the absence of their cognate effector molecules (21, 23, 36). As mentioned above, the CifR protein is predicted to be a TetR family member. Considering this data together with the demonstration that the *cifR* gene is important for repression of *cif* gene expression, we hypothesized that CifR is repressing *cif* gene expression by binding to the predicted promoter region directly upstream of the *morB*-PA2933-*cif* operon (Fig. 5A). We employed EMSA to study CifR-DNA interactions. If indeed CifR is capable of binding to the *cifR*-*morB* intergenic region, a shift in migration

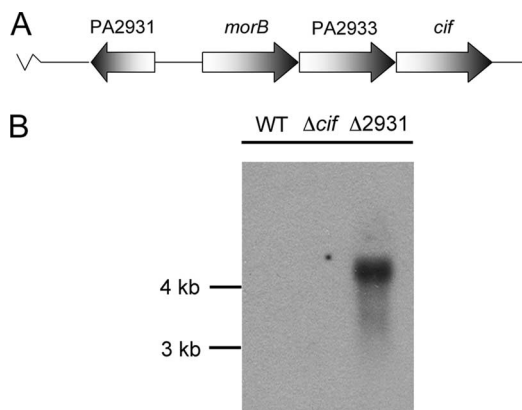


FIG. 3. *cif* is cotranscribed with *morB* and PA2933. (A) The *cif* gene is predicted to belong to a three-gene operon including *morB*, PA2933, and *cif*. (B) RNA from the wild type (WT) and the Δ*cif* and ΔPA2931 mutants was assayed using Northern blotting to determine the transcript size detected by the *cif*-specific probe. A single band at ~4.2 kb was detected in the ΔPA2931 mutant.

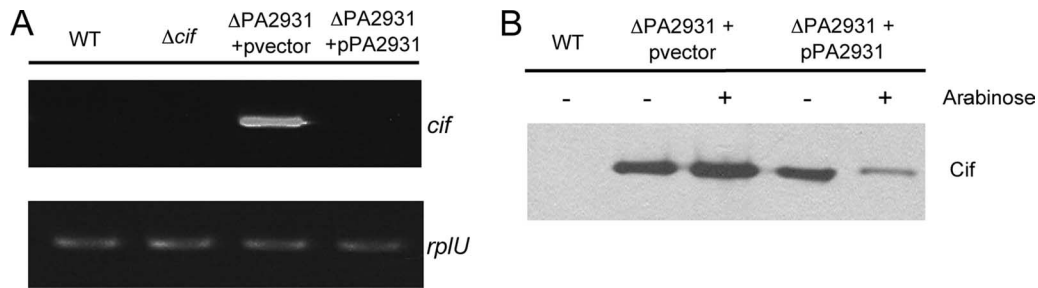


FIG. 4. *cifR* negatively regulates *cif* gene expression. *cif* gene and Cif protein expression were assayed using either sqRT-PCR or Western blotting, respectively. (A) The wild type (WT), the Δcif mutant, and the $\Delta PA2931$ mutant harboring the empty vector pMQ71 or expressing the PA2931 gene from the plasmid pDPM79 were assayed for *cif* and *rplU* gene expression. Deletion of the PA2931 gene resulted in higher levels of *cif* gene transcript, which was complemented by expression of the histidine-tagged variant of PA2931. (B) Western blot of the WT *P. aeruginosa* strain PA14, the $\Delta PA2931$ mutant containing the empty vector pMQ71, and the $\Delta PA2931$ mutant carrying a plasmid expressing a histidine-tagged variant of PA2931 from the arabinose-inducible expression plasmid pDPM79. Arabinose was added to a final concentration of 0.2% where indicated.

of the probe should be observed when CifR is incubated with the biotinylated probe.

The incubation of the purified CifR protein with the biotinylated probe resulted in a substantial mobility shift consistent with the formation of a CifR-DNA complex (lane 2, Fig. 5B, lane 2), compared to the shift with probe alone (Fig. 5B, lane 1). This binding was shown to be sensitive to the addition of the unlabeled cold probe, suggesting specificity of CifR for the intergenic region (Fig. 5B, lane 3). As an additional control, we utilized a nonspecific cold competitor derived from the *rplU* gene from *P. aeruginosa*. Inclusion of the *rplU* amplicon at a

100 \times molar excess did not result in a decrease in CifR binding to the biotinylated probe (data not shown).

The binding of CifR to the *cifR-morB* intergenic region is concentration dependent; as CifR concentration increased, we observed an increase in the CifR-DNA complex (Fig. 5C). Interestingly, under the conditions assayed, binding was observed at molar DNA-to-protein ratios as low as 1:100 (Fig. 5C, lane 2). By using a best-fit curve to determine the half-maximal effective concentration (EC_{50}) of CifR binding to the intergenic region assayed, we found that CifR has an EC_{50} of approximately 118 pM. As this system consists of only two

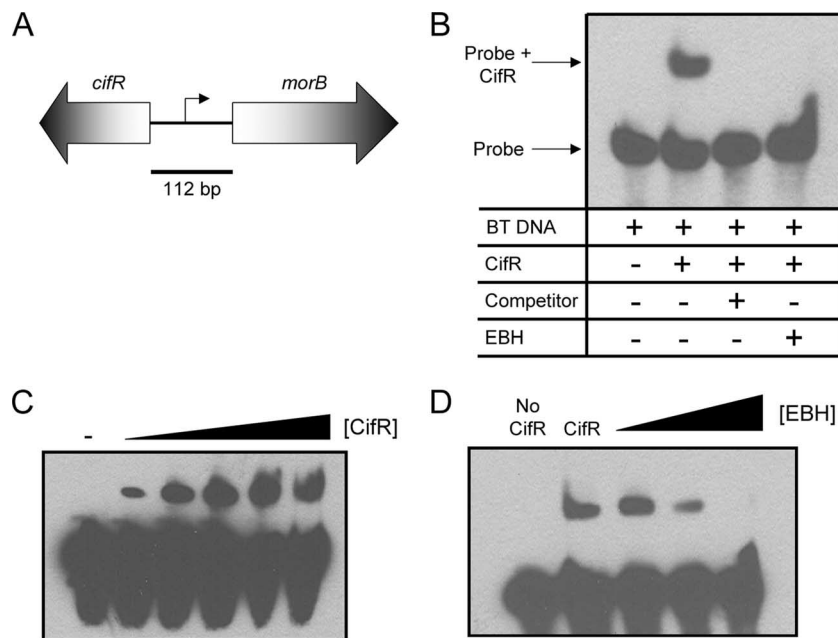


FIG. 5. CifR binds to the intergenic region directly upstream of the *cif*-containing operon. (A) The intergenic region between the *cifR* and *morB* genes was amplified using biotinylated primers and used as a probe in EMSAs. (B) EMSA using the *morB-cifR* intergenic region as a probe and purified CifR. Lane 1, labeled probe; lane 2, probe and 1.15 nM CifR; lane 3, probe, 1.15 nM CifR, and a 20 \times molar excess of the unlabeled cold competitor; and lane 4, probe, 1.15 nM CifR, and 1 mM EBH. BT, biotinylated. (C) EMSA CifR dose response. Lane 1, probe alone; lane 2, probe and 115 pM CifR; lane 3, probe and 287.5 pM CifR; lane 4, probe and 575 pM CifR; lane 5, probe and 862.5 pM CifR; and lane 6, probe and 1.15 nM CifR. (D) EMSA EBH dose response. Lane 1, probe alone; lane 2, probe and 1.15 nM CifR; lane 3, probe, 1.15 nM CifR, and 10 μ M EBH; lane 4, probe, 1.15 nM CifR, and 100 μ M EBH; and lane 5, probe, 1.15 nM CifR, and 1 mM EBH.

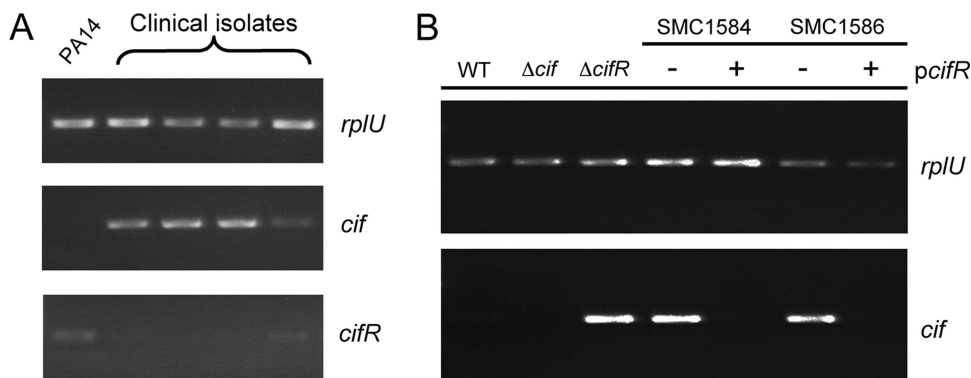


FIG. 6. Clinical isolates overexpressing the *cif* gene demonstrate decreased *cifR* gene expression. (A) sqRT-PCR of wild-type *P. aeruginosa* PA14 and four nonmucoid *P. aeruginosa* CF sputum isolates. Expression of the *cif*, *cifR*, and *rplU* genes was assayed. (B) sqRT-PCR of wild-type (WT) *P. aeruginosa*, the Δcif and $\Delta cifR$ mutants, and two of the clinical isolates from panel A carrying either the empty vector pMQ71 (-) or the *cifR*-expressing plasmid pDPM79 (+).

components, we can assume that it is noncompetitive, and thus the EC_{50} value is approximately equal to the CifR/DNA binding constant.

Based on the finding that EBH stimulates *cif* gene expression in vivo, we tested whether EBH could impact the CifR-DNA complex in vitro. The CifR-DNA complex is disrupted by the addition of EBH (Fig. 5B, lane 4). This finding is in direct agreement both with the results showing increased *cif* gene transcription in the presence of EBH and with several reports in the literature demonstrating that the presence and binding of certain ligands to TetR family protein members results in disassociation of the protein from its cognate DNA-binding site (16, 26, 39). It should be noted that EBH does not generally disrupt DNA-protein complexes, as we have tested the effects of EBH on another DNA-binding protein using EMSA and found that there was no effect on binding (data not shown).

As we showed in Fig. 2A and B, induction of *cif* gene expression and Cif protein expression by EBH is concentration dependent between 10 μ M and 1 mM. As shown in Fig. 5D, the effect of EBH on CifR DNA binding is also concentration dependent. Interestingly, inhibition of the formation of the DNA-protein complex occurs at concentrations similar to those observed for Cif induction by EBH in vivo (Fig. 2A and B).

***cif*-overexpressing clinical isolates demonstrate altered *cifR* gene expression.** A previous report from our group demonstrated that nonmucoid *P. aeruginosa* strains isolated from CF sputum demonstrate relatively high levels of *cif* gene expression compared to levels associated with the lab strain *P. aeruginosa* PA14 (29). We also found that mucoid CF isolates, those producing high concentrations of the exopolysaccharide alginate, had relatively lower levels of *cif* gene expression compared to levels associated with PA14. The striking similarity between the nonmucoid *cif*-overexpressing strains and the $\Delta cifR$ strain led us to hypothesize that the increased *cif* expression observed in these nonmucoid clinical strains was due to decreased *cifR* gene expression.

Nonmucoid *P. aeruginosa* clinical isolates previously shown to overexpress *cif* were assayed using sqRT-PCR for *rplU*, *cif*, and *cifR* gene expression. All of the clinical isolates assayed demonstrated increased *cif* expression compared to that of the

wild-type PA14 and compared to the *rplU* expression (Fig. 6A). Interestingly, all of the nonmucoid clinical isolates also demonstrated a marked decrease in *cifR* gene expression compared to that with the wild-type laboratory strain PA14. Furthermore, the one isolate that expressed slightly higher levels of *cif* (lane 5) demonstrated a concomitant decrease in *cif* expression, suggesting an inverse relationship between *cif* and *cifR* expression in *P. aeruginosa* CF sputum isolates.

To further demonstrate that the *cif* overexpression phenotype was due to a decrease in *cifR* expression, the plasmid pDPM79 expressing *cifR* was introduced into two of these strains. We found that the expression of *cifR* in the nonmucoid *cif*-overexpressing clinical strains results in a significant decrease in *cif* expression (Fig. 6B). Only two of four strains in Fig. 6A were assayed, as isolation of stable transformants of the other two strains was never achieved (data not shown).

The *cif* gene overexpression phenotypes appeared to be the result of heritable changes in gene expression, as these phenotypes were stable following serial passaging of the strains in both solid and liquid media (data not shown). We hypothesized that the loss of *cifR* expression in these clinical isolates was due to changes within the promoter region of the *cifR* gene. In order to identify any changes affecting *cifR* gene expression, we amplified the chromosomal region containing both the *cifR* promoter region and the ORF and sequenced the resulting amplicon. Surprisingly, there were no changes to the promoter region. While there were changes within the *cifR* ORF in these strains, none of these nucleotide changes were predicted to result in either missense or nonsense mutations.

DISCUSSION

We have previously demonstrated that *P. aeruginosa* produces a novel toxin, Cif, which is capable of decreasing apical membrane expression of CFTR in a variety of epithelial cell culture lines (29, 46). In silico analysis of the predicted amino acid sequence of this protein suggested that it may belong to the large family of α/β hydrolases and in particular to the family of EHs. Supporting this hypothesis, Cif was shown to degrade the general model EH substrate *S*-NEPC.

The ability to degrade *S*-NEPC, while suggestive, was not

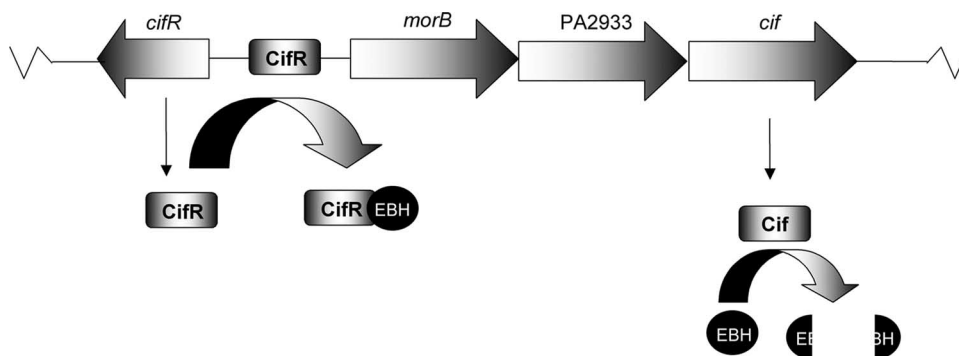


FIG. 7. Model for *cif* gene expression. CifR represses *cif* gene expression by binding to the promoter region immediately adjacent to the *cif*-containing operon. The binding of EBH by CifR results in disassociation of the repressor from the promoter, resulting in increased *cif* gene expression and biosynthesis of the EH Cif, which in turn degrades EBH.

conclusive evidence that Cif is an EH. In order to better understand the biological activity of Cif, i.e., decreased apical membrane expression of CFTR, we sought to further characterize the biochemical activity of this protein. Previous work identified a soil pseudomonad capable of degrading the epoxide EBH (24). This previous study demonstrated that this EH activity was the result of a secreted protein with a mass and amino acid sequence similar to those of the Cif protein, leading us to hypothesize that Cif may be a homolog of the EH described previously that would thus be capable of degrading EBH. The finding that Cif could indeed degrade EBH supported this hypothesis. Furthermore, the demonstration that EBH could significantly induce *cif* gene expression supports the conclusion that this family of molecules can serve as substrates for Cif.

Epoxides have been previously shown to act as signaling molecules regulating processes as varied as Cl^- ion transport in renal tubular epithelial cells, angiogenesis, and vasodilation mediated by endothelial cells (32, 33, 42). Furthermore, several studies have demonstrated the presence of high levels of leukotrienes within the CF airway. Leukotrienes are produced from the inactive epoxide molecule leukotriene A₄, which is metabolized by the EH leukotriene hydrolase to the active, vicinal diol form, leukotriene B₄. Leukotriene B₄ has been shown to be a potent chemoattractant, specifically for neutrophils, and is generally considered a mediator of inflammation (10, 32, 34, 50, 51). It still remains unclear how Cif alters apical membrane expression of CFTR, but it may be through the metabolism of these epoxide signaling molecules. Thus, CifR may regulate *cif* gene expression, and hence Cif biosynthesis, by binding of native epoxide molecules in the CF lung. These molecules have yet to be identified. While we do not believe that EBH, an industrial pollutant, is found in the CF lung, or acts as a signaling molecule within the CF lung, it serves as a model compound for better understanding the regulation of *cif* gene expression by CifR.

The family of TetR repressors has been shown to regulate the expression of genes involved in a variety of pathways, from antibiotic resistance to carbon catabolism (20, 23, 36). Typically TetR family members have a DNA-binding helix-turn-helix (HTH) motif as well as an effector-binding domain. The protein acts to regulate gene expression by binding to the promoter region of its target gene and thus inhibiting tran-

scription. The presence of an effector causes conformational shifts in the HTH domain, resulting in disassociation of the protein from the promoter and hence derepression of gene expression (16, 21, 26, 36). Comparison of the CifR amino acid sequence to known TetR family members showed a similar organization, including a HTH DNA-binding domain and a predicted effector-binding domain with no homology to any known TetR protein. We have shown here that the CifR protein is capable of binding to the predicted promoter region of the *cif*-containing operon and that this interaction is sensitive to the epoxide EBH (Fig. 7). These data readily explain the EBH-dependent induction in *cif* gene expression shown in Fig. 2A. Interestingly, we found that this disassociation was concentration dependent and that this phenomenon occurred at concentrations similar to those shown in Fig. 2A, indicating that the concentration of EBH used to disrupt the DNA-protein complex in the *in vitro* studies is physiologically relevant.

In silico analysis of the *cifR-morB* intergenic region has identified a putative CifR-binding site consisting of two palindromic sequences that overlap both the putative -10 and -35 sequences of the *morB* and *cifR* promoters. Work is ongoing to identify and characterize the CifR-binding site. Interestingly, the putative promoter regions of the *morB* and *cifR* genes overlap significantly, as does the putative CifR-binding site. Thus, most likely, CifR not only represses expression of the *cif*-containing operon but also negatively regulates its own expression in the absence of EBH.

We have previously demonstrated that nonmucoid *P. aeruginosa* strains isolated from CF sputum demonstrated significantly increased *cif* gene expression compared to that with the *P. aeruginosa* strain PA14 (29), while mucoid CF isolates displayed lower levels of *cif* gene expression. These observations led us to hypothesize that the differential *cif* gene expression was the result of changes in expression of CifR. In support of this hypothesis, we found that in several of the nonmucoid strains overexpressing *cif*, *cifR* gene expression was significantly lower than the expression seen in the laboratory strain PA14. Furthermore, when CifR was expressed from the plasmid pDPM79 in two of the *cif*-overexpressing clinical isolates, we observed a level of *cif* transcript that was significantly lower than that with the vector control strains. Interestingly, the *cif* overexpression phenotype of nonmucoid clinical isolates was

stable, as repeated passaging of the strains in laboratory media did not effect *cif* gene expression, suggesting that these strains had acquired mutations either in the *cifR* promoter region or within the ORF itself. However, DNA sequence analysis of the *cifR* promoter region did not illuminate any mutations in the promoter. We did find several mutations within the ORF, although none of these are predicted to result in either missense or nonsense mutations. Several models for the decreased expression of *cifR* in these strains can be invoked, including changes in mRNA stability, structural changes in the mRNA, or changes in genes that regulate *cifR* expression. Currently, we believe the latter to be the case, and studies are ongoing to identify regulators of both *cif* and *cifR* gene expression.

It is generally believed that CF patients are initially colonized by nonmucoid environmental isolates, which eventually convert to the mucoid phenotype (4, 15, 35, 43). These data, taken together with our demonstration that nonmucoid clinical isolates show relatively high *cif* expression compared to that of mucoid clinical isolates, suggest that Cif may play a role in initial colonization of the CF lung and early infection. Classically, TetR family regulators have been shown to regulate gene expression as a response to environmental stimuli, as is the case with CifR and its response to EBH. We hypothesize that CifR may respond to the presence of an endogenous epoxide and regulate *cif* gene expression accordingly, thus affecting CFTR membrane expression through an as-yet-unidentified mechanism.

An alternative hypothesis is that there is a selective pressure for strains that overexpress *cif* in the context of the CF lung and that this pressure leads to a population that has increased *cif* expression as a result of decreases in *cifR* expression. The demonstration that the *cif*-overexpressing phenotype in clinical isolates was heritable and stable in these strains suggests that this phenotype is due to genotypic changes altering *cifR* gene expression. Thus, rather than sensing the environment and responding through classic regulatory pathways, it would appear that *P. aeruginosa* in the CF lung may simply remove the regulatory elements for some genes, thus altering their basal gene expression.

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