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Characterization of Interactions between the Transcriptional Repressor PhlF and Its Binding Site at the *phlA* Promoter in *Pseudomonas fluorescens* F113

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The *phlACBD* genes responsible for the biosynthesis of the antifungal metabolite 2,4-diacetylphloroglucinol (PHL) by the biocontrol strain *Pseudomonas fluorescens* F113 are regulated at the transcriptional level by the pathway-specific repressor PhlF. Strong evidence suggests that this regulation occurs mainly in the early logarithmic phase of growth. First, the expression of the *phlF* gene is relatively high between 3 and 13 h of growth and relatively low thereafter, with the *phlACBD* operon following an opposite expression profile. Second, the kinetics of PHL biosynthesis are specifically altered in the logarithmic phase in a *P. fluorescens* F113 *phlF* mutant. The *phlA-phlF* intergenic region presents a complex organization in that *phlACBD* is transcribed from a σ^{70} RNA polymerase-dependent promoter that is likely to overlap the promoter of the divergently transcribed *phlF* gene. The repression by PhlF is due to its interaction with an inverted repeated sequence, *phO*, located downstream of the *phlA* transcriptional start site. Cross-linking experiments indicate that PhlF can dimerize in solution, and thus PhlF may bind *phO* as a dimer or higher-order complex. Furthermore, it is now demonstrated that certain regulators of PHL synthesis act by modulating PhlF binding to *phO*. PHL, which has previously been shown to be an autoinducer of PHL biosynthesis, interacts with PhlF to destabilize the PhlF-*phO* complex. Conversely, the PhlF-*phO* complex is stabilized by the presence of salicylate, which has been shown to be an inhibitor of *phlA* expression.

2,4-Diacetylphloroglucinol (PHL) is a phenolic molecule produced by many fluorescent pseudomonad bacteria (3, 23, 42, 47, 55, 56, 61, 63). It has antifungal (28, 55, 58), antibacterial (28), antihelminthic (7, 21), and phytotoxic (23, 48) activities. PHL is a polyketide synthesized by condensation of three molecules of acetyl coenzyme A with one molecule of malonyl coenzyme A to produce the precursor monoacetylphloroglucinol, which is subsequently transacetylated to generate PHL (37, 55).

The genes involved in the biosynthesis of PHL have been cloned and sequenced from a number of *Pseudomonas* strains (3, 4, 11, 17, 37, 55, 61). Genetic and sequence data show that the *phl* locus is comprised of a number of transcriptional units. The structural genes, *phlA*, *phlC*, *phlB*, and *phlD*, are transcribed as a single operon (*phlACBD)*, with the putative permease gene, *phlE*, probably transcribed from its own promoter further downstream. The transcriptional repressor, *phlF*, is located upstream of the *phlACBD* operon and is transcribed in the opposite direction (4, 11, 54). This organization is common to many polyketide biosynthetic loci (14, 41, 43).

There is evidence that PHL production is influenced by environmental and nutritional signals as well as by transcriptional and posttranscriptional regulatory systems. The environmentally responsive GacS/GacA two-component regulatory system is required for production of PHL, with mutation of either component completely abolishing PHL biosynthesis (10, 11, 15). Transcriptional studies have demonstrated that the GacS/GacA system positively regulates expression of both *phlACBD* and *phlF* (11, 20). At another level, sigma factors may also play a role in regulation at the *phl* locus. It has been shown that overexpression of the housekeeping sigma factor *rpoD*, encoding σ^{70}, or mutation of the stationary-phase sigma factor *rpoS* increased PHL production (38, 53, 64).

Pathway-specific regulators are also involved in regulation of PHL biosynthesis. The *phl* biosynthetic gene cluster is negatively regulated by the repressor PhlF and positively modulated by the genetically linked *phlH* gene, which encodes a putative regulator (11, 54). Posttranscriptional control via homologues of the *Escherichia coli* CsrA and CsrB molecules also plays a role in controlling PHL production. This system, which comprises a translational repressor protein (CsrA/RsmA) and a regulatory RNA (CsrB/RsmB), has been described in a number of gram-negative bacteria (6, 30–33, 36, 50). Recently, we reported the characterization of the *prrB* gene encoding a regulatory RNA that is a functional homologue of CsrB/RsmB. Overexpression of *prrB* RNA restores PHL production in *gacA* and *gacS* mutants and leads to overproduction of PHL in a wild-type *P. fluorescens* strain (1).

It has been established that PHL is an autoregulator, positively influencing its own production. Furthermore, salicylate and other secondary metabolites (fusaric acid and pyoluteorin) have a negative effect on PHL production. In both cases, an intact *phlF* gene is required, suggesting that PHL and salicylate act via PhlF (54). Thus, the interaction of PhlF with coregula-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Pseudomonas fluorescens F113	Wild-type; HCM^+ Prt^+ Lac^-	55
Escherichia coli DH5α	ϕ80 *lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 hsdR17 recA1 endA1 thi-1*	52
Plasmids		
pMP190	Promoterless *lacZ* vector, IncQ Cm^r Sm^r	57
pMP220	Promoterless *lacZ* vector, IncP Tc^r	57
pQE-60.27	700-bp *Nco*I-*Bgl*II fragment containing PCR-amplified *phlF* in pQE-60	12
pCU107	*phACBD-lacZ*, 5-kb *Eco*RI fragment from pCU203 in pMP220, Tc^r	12
pCU109	*phlF-lacZ*, 1.8-kb *Sal*I-*Kpn*I fragment from pCU203 in pMP190, Cm^r	12

tors and the *phlACBD* promoter plays an important role in regulating PHL production. This paper describes the characterization of the promoter of the *phlACBD* gene cluster and identification of the PhlF binding site (operator). It is also established that the autoinducer (PHL) and the corepressor (salicylate) interact physically with the PhlF repressor to modulate PhlF binding to its operator sequence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *P. fluorescens* F113 and derivatives were routinely grown at 28°C in Luria-Bertani (LB) broth and in minimal medium (SA) with sucrose (50 mM) and asparagine (17.5 mM), respectively, as sole carbon and nitrogen sources. SA medium was supplemented with 100 μM $FeCl_3$. *E. coli* strains were grown at 37°C in LB broth (52). Antibiotics were used at the following concentrations: for *P. fluorescens*, tetracycline, 75 μg/ml, and chloramphenicol, 250 μg/ml; for *E. coli*, tetracycline, 25 μg/ml, chloramphenicol, 30 μg/ml, and ampicillin, 100 μg/ml.

Recombinant DNA techniques. Small-scale and large-scale plasmid DNA isolations were performed using Qiagen plasmid mini and maxi kits, respectively, according to the manufacturer's specifications (Qiagen, Inc.). Plasmids were introduced into *E. coli* and *P. fluorescens* by electroporation (16) or mobilized into *P. fluorescens* by triparental matings using the helper plasmid pRK2013 (19).

Construction of transcriptional fusions and β-galactosidase assays. To characterize the transcriptional activity of the *phlACBD* and *phlF* genes, we used pCU107 and pCU109 constructs bearing *lacZ* transcriptionally fused with *phlACBD* and *phlF*, respectively (11, 12). The kinetics of expression of biosynthetic fusions in different backgrounds were monitored by performing β-galactosidase assays (40). *P. fluorescens* F113 cells were grown with shaking (150 rpm) in 100-ml flasks containing 25 ml of medium. All measurements were performed in triplicate. For time course experiments, samples were taken from cultures at the indicated times.

RNA techniques. Total RNA was isolated from 7×10^9 cells of wild-type *P. fluorescens* F113 grown for 18 h on LB broth with the RNeasy total RNA isolation kit according to the manufacturer's specifications (Qiagen, Inc.). RNA integrity was checked by electrophoresis in 1.2% (wt/vol) agarose gels containing 0.66% (vol/vol) formaldehyde. Primer extension was performed by the method of Pujic et al. (44) with the following modifications: 250 pmol of primer PE-A, which hybridized 71 nucleotides downstream of the ATG translation start codon of *phlA*, was end labeled using T4 polynucleotide kinase and [γ^{32}P]ATP. Labeled primers and total RNA were hybridized at 65°C for 5 min and allowed to cool at room temperature for 1.5 h. Reverse transcription was performed at 42°C for 1 h using avian myeloblastosis virus reverse transcriptase. Sequence reactions were performed with the same primers using *P. fluorescens* F113 plasmid DNA as a template.

Purification of PhlF. We have previously reported the purification of a histidine-tagged PhlF repressor (23.6 kDa) (12). A protein of smaller size (18 kDa) invariably copurified with the histidine-tagged PhlF protein. This 18-kDa protein is thought to be a derivative of PhlF truncated by approximately 50 amino acids at its N terminus. Pure PhlF protein was needed in this study for two reasons: first, to avoid any possible competition between the two proteins in the binding assays, especially when analyzing the influence of PHL on PhlF binding activity, and second, to avoid any interference between the functional full-sized PhlF and the truncated form in cross-linking experiments. For these reasons we optimized the purification conditions to purify only the full-length PhlF. A number of parameters were modified relative to the previous protocol (12). The procedure is detailed below, the major modifications being the use of a different strain of *E. coli* and different inducing conditions.

The PhlF-overexpressing plasmid pQE-60.27 was introduced into *E. coli* DH5α by electroporation. Then 300 ml of LB broth containing ampicillin (100 mg/ml) was inoculated at an absorbance at 600 nm of 0.1 by an overnight culture of DH5α/pQE-60.27 and grown at 37°C with shaking (200 rpm) to an optical density at 600 nm (OD_{600}) of 0.5. Overexpression of *phlF* was induced by addition of 0.2 mM isopropylthiogalactopyranoside (IPTG). After incubation at 30°C for 4.5 h, the cells were harvested by centrifugation ($5,000 \times g$) for 15 min at 4°C, washed twice in LB broth, and resuspended in 4 ml of lysis buffer (50 mM Na_2PO_3, 300 mM NaCl, 10 mM imidazole, 40 mg of lysozyme). Cells were then incubated on ice for 15 min and passed twice though a French press. Cell debris was removed from the crude extract by centrifugation ($10,000 \times g$) for 40 min at 4°C, and the 6xHis-tagged PhlF protein was purified under nondenaturing conditions using Ni-nitrilotriacetic acid (Ni-NTA) spin columns, following the manufacturer's protocol (Qiagen, Inc.). The purified PhlF-6xHis was then dialyzed overnight at 4°C against 1× TE (Tris-EDTA) buffer.

Gel retardation. DNA fragment 7EH of 145 bp covers the common sequence between 2.9 and 7.U used by Delany et al. (12). This fragment of the intergenic region was amplified using specific primers 7A5E and 7A3H with pCU109 as the DNA template. Four double-stranded oligonucleotides, p12d, p11d, p10d, and p9d, of 35 bp each were obtained by annealing oligonucleotides p12 and p12c; p11 and p11c; p10 and p10c, and p9 and p9c, respectively. Each double-stranded oligonucleotide overlaps the oligonucleotides adjacent to it by 4 bp. The four oligonucleotides cover the full length of the overlap between probes 2.9 and 7.U. The probes were 5′ end labeled with [γ^{32}P]CTP using polynucleotide kinase (NEB). The labeled probes were extracted once with phenol-chloroform and ethanol precipitated. The sequences of the oligonucleotides used for PCR amplification and gel retardation will be provided upon request.

Gel shift assays were performed as described by Liu et al. (34). The binding reaction was performed in 40 μl of binding buffer (12 mM HEPES-NaOH [pH 7.9], 4 mM Tris-HCl [pH 7.9], 75 mM KCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 1.0 mM dithiothreitol) containing 1 μg of salmon sperm DNA, 50 ng of poly(dI-dC), 2 μg of bovine serum albumin, 1 μl of probe, and 3 μg of purified PhlF-6xHis protein. The reaction mixture was incubated for 20 min at room temperature and directly subjected to polyacrylamide gel electrophoresis (PAGE) in a 5% (wt/vol) polyacrylamide gel containing 2.5% (vol/vol) glycerol in TAE (Tris-acetate-EDTA) buffer (40 mM Tris-acetate [pH 7.5], 2 mM EDTA). In general, electrophoresis was performed at 110 V for 2.5 h. When probes p12d, p11d, p10d, and p9d were used, electrophoresis was performed at 80 V for 1.5 h. Gels were dried and examined by autoradiography with X-ray film (Biomax; Kodak).

To analyze the effects of PHL and salicylate on PhlF binding activity, a set of solutions with different concentrations of the two metabolites were prepared in ethanol (70%). To investigate whether ethanol interferes with PhlF activity, a mobility shift assay was monitored in the presence of different concentrations of ethanol. We found that PhlF binding activity was not altered by ethanol concentrations up to 6% (data not shown). Note that the highest concentration of ethanol routinely used in binding assays with PHL and salicylate was less than 4%. As a control to determine the specificity of the effect of PHL on PhlF binding, a control band shift was performed. This used the cI2009 repressor of lactococcal bacteriophage TUC2009, which represses transcription from the early lytic promoter P_R located in the cI2009-CRO2009 intergenic region (27, 59).

Footprinting. Chemical footprinting of the PhlF-DNA complex was performed using a combined gel retardation–1,10-phenanthroline–copper ion footprinting procedure (24). Briefly, labeled probe 7EH was digested with *Eco*RI, extracted once with phenol-chloroform, and ethanol precipitated. Digested probe was resuspended in water, and gel retardation was then performed as described

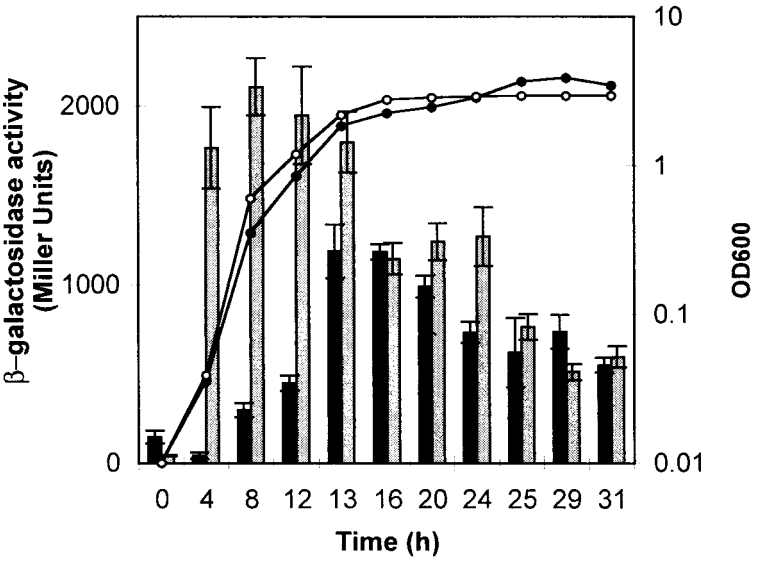

FIG. 1. Time course of expression of the *phl* operon. The transcriptional fusions *phlACBD-lacZ* (pCU107) and *phlF-lacZ* (pCU109) were introduced into *P. fluorescens* F113. Cultures were grown on minimal medium, and absorbance was measured at 600 nm (solid circles, *phlF-lacZ*; open circles, *phlACBD-lacZ*). Expression of the fusions was assessed by measuring levels of β-galactosidase. Grey shading represents *phlF-lacZ* expression, and dark shading represents *phlACBD-lacZ* expression. Triplicate cultures were assayed. The standard deviations are represented with error bars.

above. The full retardation gel was soaked in 200 ml of 50 mM Tris-HCl (pH 8.0) and treated for 10 min at room temperature with 10 ml of solution A (2 mM 1,10-phenanthroline, 0.45 mM $CuSO_4$) and 10 ml of solution B (58 mM 3-mercaptopropionic acid). The reaction was then quenched by addition of 20 ml of 28 mM 2,9-dimethylphenanthroline for 2 min. The gel was rinsed with distilled water and exposed to X-ray film for 30 min at room temperature. Bands of interest were cut and eluted overnight at 37°C in 500 mM ammonium acetate–1 mM EDTA. Eluted DNA was ethanol precipitated and resuspended in 80% (vol/vol) formaldehyde–10 mM NaOH–1.0 mM EDTA–0.1% (wt/vol) bromophenol blue. The DNA sample was run on an 8% (wt/vol) polyacrylamide sequencing gel.

Glutaraldehyde cross-linking of PhlF. The glutaraldehyde cross-linking procedure was modified from Derré et al. (13). PhlF (3 μg) was incubated with 10 mM cross-linking reagent glutaraldehyde in 40 μl of a reaction mixture containing 50 mM NaH_2PO_4, 50 mM NaCl, and 10% (vol/vol) glycerol. After incubation for 1 h at 30°C, the reaction was stopped by the addition sodium dodecyl sulfate (SDS) loading buffer. The samples were boiled and analyzed by SDS-PAGE on 10% (wt/vol) polyacrylamide gels (25). The gel was stained with Coomassie blue.

RESULTS

Kinetics of *phlA* and *phlF* gene expression. Delany et al. (12) established that wild-type *P. fluorescens* F113 does not produce PHL during early stages of growth. In a *phlF* mutant, however, the kinetics of PHL production were altered, such that PHL was produced earlier in the growth cycle. From these data, it was concluded that PhlF acts as a repressor of PHL production during the early log phase (12).

To further investigate the mechanism of regulation of PHL biosynthesis by PhlF, we performed a time course experiment monitoring the expression of *phlF* and *phlACBD* using transcriptional fusions. *P. fluorescens* F113 strains containing the *phlACBD-lacZ* transcriptional fusion carried by pCU107 and the *phlF-lacZ* transcriptional fusion carried by pCU109 were grown independently on SA medium and analyzed by Miller assays (Fig. 1). Expression of the *phlACBD-lacZ* construct was low between 0 and 12 h and reached its maximum at 13 to 20 h, when the culture was in stationary phase. This correlates with the peak of PHL production (12). In contrast, expression of the *phlF-lacZ* construct was highest earlier in the growth cycle, with maximal expression during logarithmic growth (3 to 13 h). These results are consistent with the hypothesis that the low transcriptional activity of *phlACBD* in the early logarithmic phase is a consequence of high levels of the PhlF repressor.

Characterization of the *phlA* promoter and analysis of the organization of the *phlA-phlF* intergenic region. To better understand how PhlF influences the transcriptional activity from the *phlA* promoter, we decided to characterize the transcriptional start of the *phlACBD* operon. Total RNA isolated from wild-type *P. fluorescens* F113 grown for 18 h was subjected to primer extension analysis. This analysis revealed only one specific transcript starting 363 bp upstream of the translational start (Fig. 2). A putative σ^{70} −10 element (TAGGAT) and a perfectly conserved −35 element (TTGACA) were identified (Fig. 3). The sequences of the two promoter elements are perfectly conserved in two other strains of *P. fluorescens*, Q2-87 (U41818) and CHA0 (AF207529). Similar promoter organization has already been reported for a number of genes in *Pseudomonas aeruginosa* (35, 39).

Detailed analysis of the *P. fluorescens phlA-phlF* intergenic region revealed the presence of many inverted repeat sequences. Interestingly, in both strain F113 and strain Q2-87, the −10 element is located between inverted repeat sequences (Fig. 3). This could indicate that access of RNA polymerase to the −10 box is regulated. The sequence CCAAT, which represents a common motif of low-temperature-induced genes in *E. coli* and other bacteria (26, 45, 51), is perfectly conserved at position −40 from the transcriptional start in strains F113 and Q2-87 but not in strain CHA0. The sequence data suggest that the *phlA* gene is transcribed from a σ^{70}-dependent promoter in

FIG. 2. Primer extension analysis mapping the *phlA* transcriptional start site. Primer extension (PE) on total RNA from *P. fluorescens* F113 was performed using primer PE-A, which maps inside the *phlA* coding region. Plasmid DNA was sequenced using the same primer. The gel shows the only primer extension stop detected by this analysis.

all three *Pseudomonas* strains and indicate that the *phl* loci in strains F113 and Q2-87 are structurally more similar to each other than to the *phl* locus in strain CHA0. This is consistent with a recent phylogenetic analysis which established that *P. fluorescens* strains F113 and Q2-87 are more closely related to each other than to *P. fluorescens* CHA0 (46). Previously, it has been reported that PHL accumulates to higher levels at reduced temperatures, but it remains to be determined whether the CCAAT motif is involved in this phenomenon (55).

Identification of the PhlF binding site. Previously, we established that PhlF binds to the *phlA-phlF* intergenic region and used gel mobility shift analyses to localize the binding site to a 100-bp sequence (12). In order to define the precise PhlF binding site, a more comprehensive gel shift analysis was undertaken using shorter DNA probes (Fig. 4). Fragments 2.9 and 7.U were previously determined to contain the PhlF binding site. A DNA fragment, 7EH, that spanned the overlap between these probes was constructed and analyzed by gel retardation (Fig. 5). PhlF-6xHis, used in the mobility shift assays, was purified according to the modified protocol described in Materials and Methods. It is clearly seen that the migration of fragment 7EH was retarded upon addition of PhlF. No retarded band was seen when a large excess of unlabeled 7EH DNA fragment was added along with PhlF protein to the 7EH labeled fragment.

To more precisely localize the PhlF binding site, we designed internal double-stranded primers, p12d, p11d, p10d, and p9d, spanning most of the 7EH region (Fig. 4). Mobility shift experiments established that only p12d was able to bind PhlF (data not shown). Probe p12d contains an inverted repeated sequence of eight bases separated by eight bases that we designate the *phl* operator, *phO* (Fig. 3). It was noted that the strength of binding of PhlF to probe 7EH appeared to be stronger than to probe p12d (data not shown).

To address the possibility that weaker interactions between nucleotides within probe 7EH and PhlF stabilize or enhance binding of PhlF to the operator sequence *phO*, the PhlF footprint on probe 7EH was identified (Fig. 6). It was found that binding of PhlF to 7EH protected the three regions of the probe, designated boxes 1, 2, and 3. Box 1 is a 30-bp stretch of DNA that contains the inverted repeat sequence that has been designated the *phO* operator (Fig. 3). Boxes 2 and 3, 9 and 7 bp, respectively, are shorter stretches of DNA (Fig. 3). Box 2 is contained within probe p10d, and box 3 is contained within probe p9d, and since neither of these probes can bind PhlF, it is concluded that boxes 2 and 3 do not constitute primary PhlF binding sites. Interaction of PhlF with these boxes may, however, contribute to the stabilization of PhlF bound to the *phO* operator (box 1). Further support for the premise that *phO* constitutes the PhlF binding site came from analysis of mutations between the inverted repeat regions. Any sequence changes in this region abolished the ability of PhlF to bind the *phO* operator (data not shown).

By analogy to other DNA-binding proteins (13, 29), the structure of *phO* suggests that the PhlF repressor may be active as a dimer or even a multimer. Dimerization of the PhlF was investigated in vitro by chemical cross-linking with a bifunctional reagent, glutaraldehyde (13). It was found that un-cross-linked PhlF migrates at 30 kDa (theoretical mass of PhlF is 23.6 kDa) and that incubation of a PhlF monomer solution

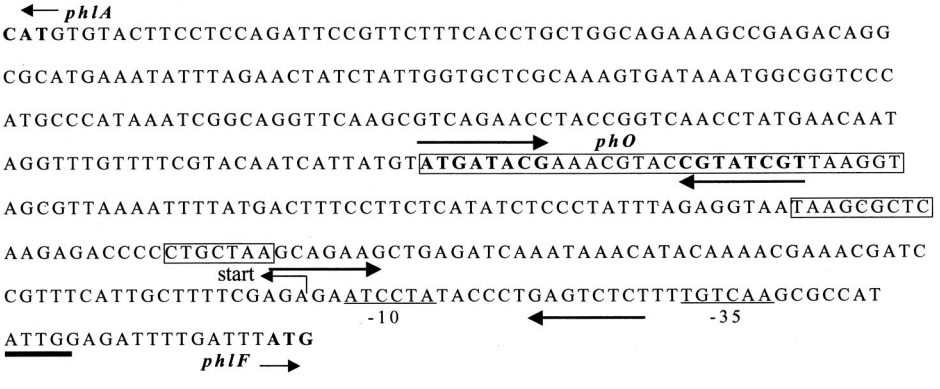

FIG. 3. Sequence organization of the *phlA-phlF* intergenic region. *phlA* and *phlF* indicate the start of the *phlA* and *phlF* open reading frames, respectively. Start indicates the transcriptional start of the *phlACBD* operon. The putative σ^{70} −10 and −35 elements for *phlA* are underlined. The PhlF binding site, *phO,* is indicated, with its two inverted repeated sequences shown by arrows. The three regions protected by the PhlF footprint are boxed. From 5′ to 3′, these are box 1, box 2, and box 3, as referred to in the text. The CCAAT (ATTGG) motif of low-temperature-induced genes is underlined. The inverted repeated sequences flanking the −10 element are indicated with arrows. The accession number for this sequence is AF497760.

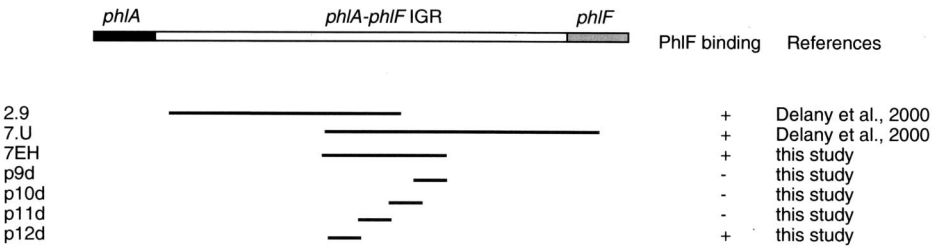

FIG. 4. Map of probes used in the mobility shift assays in this study. The names of probes are presented on the left of the figure. Binding of PhlF protein to the probes is indicated as + or −. IGR, intergenic region. The 5′ ends of the *phlA* and *phlF* ORFs are indicated

with glutaraldehyde generated an altered form of PhlF with reduced electrophoretic mobility (≈63 kDa) (Fig. 7). These results suggest that PhlF can dimerize in solution and may bind to the *phO* operator as a dimer. Although in vitro evidence of higher-order protein complexes was not obtained, it remains a possibility that the protein may bind DNA as a larger complex, for example, a tetramer.

Effect of PHL and salicylate on PhlF binding activity. Recently, it was reported that PHL induced and salicylate repressed *phlA* expression via PhlF (54). It was not established how these metabolites influenced PhlF activity. To determine whether PHL could modulate binding of PhlF to the *phO* operator, PHL was added in increasing concentrations to a binding mixture containing the radiolabeled probe 7EH and PhlF (Fig. 8A). It was found that PHL reduced PhlF binding in a concentration-dependent fashion. This suggests that there is a physical interaction between PHL and PhlF. This effect was independent of the order in which the components were added, indicating that PHL may dissociate preformed PhlF-*phO* complexes. An alternative explanation is that there may be an equilibrium between free and bound PhlF protein. These data demonstrate that autoinduction of *phlACBD* transcription by PHL operates by reducing binding of the repressor PhlF to its operator. The specificity of PHL for PhlF was demonstrated by performing a gel shift assay using the repressor protein cI2009 of phage Tuc2009 and a DNA fragment containing its binding site (59). cI2009 binding activity was not affected by 3 mM PHL (Fig. 8B).

Salicylate enhances the repression of *phlA* expression by PhlF in vivo (54). To investigate whether this is due to a direct interaction with the repressor, binding of PhlF to the *phO* operator in the presence and absence of salicylate (4 mM) was investigated. Band shift assays were performed over a range of PHL concentrations, and the relative concentrations of bound and unbound probe were determined (Fig. 9). It was found that the presence of salicylate results in longer preservation of the PhlF-probe complex even at high concentrations of PHL.

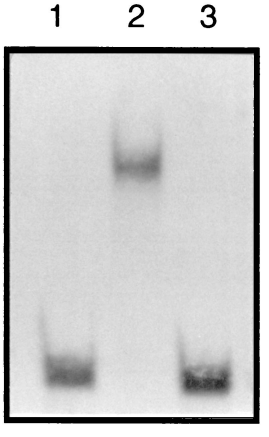

FIG. 5. Identification of the PhlF binding site. Probe 7EH (2 ng) was analyzed for reduced mobility in a native gel in the presence of 3 μg of purified PhlF. Lane 1 contains labeled 7EH probe; lane 2 contains labeled 7EH probe and 3 μg of PhlF-6xHis; lane 3 contains labeled 7EH probe, 3 μg of PhlF-6xHis, and an excess of unlabeled 7EH probe.

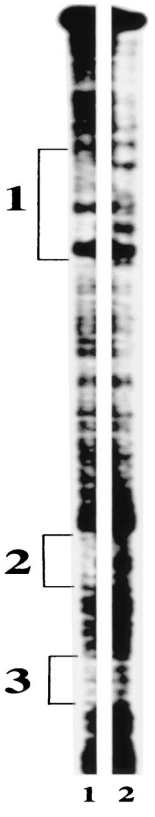

FIG. 6. Footprint of PhlF. DNA probe 7EH that was bound by PhlF (lane 1) or unbound (lane 2) was treated with a chemical footprinting agent to locate the PhlF footprint. Three protected regions of the probe were identified and labeled 1, 2, and 3. The positions of these protected regions are shown in Fig. 3.

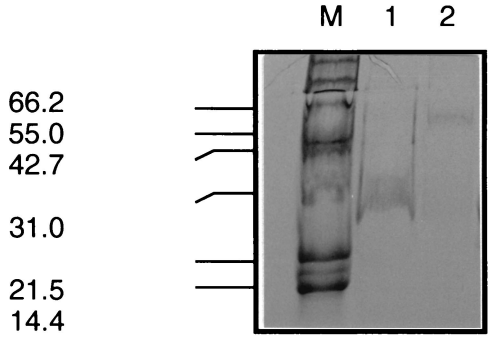

FIG. 7. PhlF forms a dimer in vitro. SDS-PAGE analysis of purified PhlF-6xHis samples (3 μg) without (lane 1) and with (lane 2) treatment with 10 mM glutaraldehyde. Midrange molecular size standards were loaded the left lane; sizes are shown in kilodaltons.

The same effect of salicylate was observed independent of the sequence in which the components of the binding reaction were added. These data indicate that salicylate interacts physically with PhlF.

DISCUSSION

Control of *phlACBD* expression by PhlF. Previous studies reported that *P. fluorescens* does not produce PHL in early log phase (12, 54). In the present study, we have demonstrated that this is likely due to the high expression of the *phlF* repressor gene during this growth phase. This is supported by our previous finding that in the *P. fluorescens* F113 *phlF* mutant, PHL production is derepressed mainly in the early stage of growth (12). It is noteworthy, however, that even in the early stages of growth, when *phlF* expression is relatively high, *phlACBD* transcription is not completely absent. Although this indicates that there is not complete repression of *phlACBD* transcription by PhlF, PHL is not detected during this phase of growth. This is consistent with the view that PHL biosynthesis is regulated at more then one level (1).

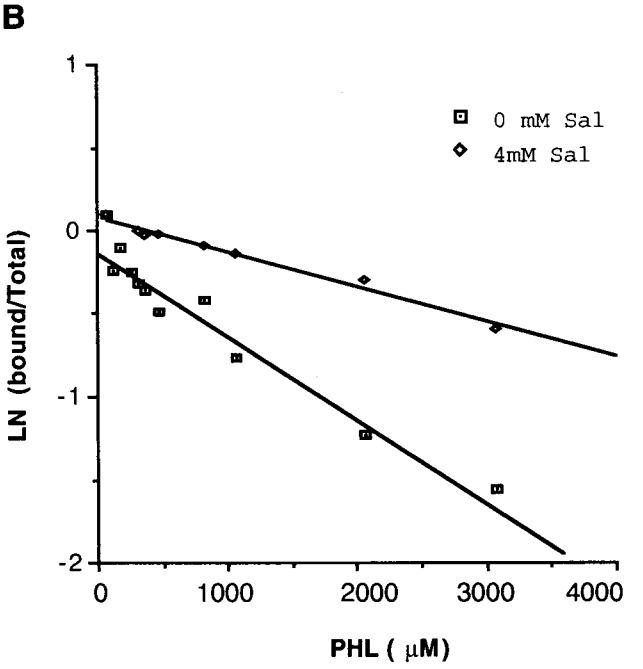

FIG. 9. Salicylate enhances PhlF binding to *phO*. Binding reaction mixtures containing 2 ng of radiolabeled 7EH probe, 3 μg of PhlF-6xHis, and increasing amounts of PHL were prepared with or without 4 mM salicylate. Binding was measured by densitometry, and the relative PhlF binding activity in the presence and absence of salicylate was calculated for each concentration of PHL. This is presented as the natural log of the intensity of bound probe divided by the total intensity of the probe. Sal, salicylate.

To understand the mechanism by which PhlF represses *phlA* transcription, it was important to determine the transcriptional

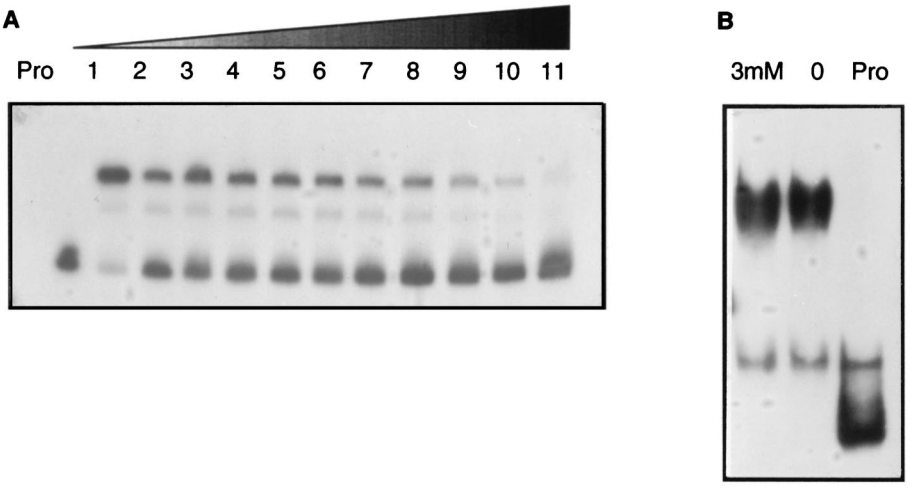

FIG. 8. (A) Impact of PHL on the electrophoretic mobility of the PhlF-7EH probe complex. Increasing amounts of PHL were added to the binding mixture containing 3 μg of PhlF-6xHis and 2 ng of radiolabeled 7EH probe. Pro, free 7EH probe. Samples 1 to 11 contain 0 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.25 mM, 0.3 mM, 0.4 mM, 0.75 mM, 1 mM, 2 mM, and 3 mM PHL, respectively. (B) Mobility shift assay using an unrelated system composed of repressor protein cI2009 of phage Tuc2009 and a DNA fragment (2880 to 3030) from the genetic switch of Tuc2009. Lanes: Pro, probe without repressor; 0, probe with repressor cI2009; 3 mM, probe with repressor cI2009 and PHL (3 mM).

start of this gene and consequently to characterize its promoter. Our analysis suggests that the *phlACBD* operon is transcribed from a σ^{70}-dependent promoter. This conclusion is supported by the fact that overexpression of the housekeeping sigma factor *rpoD* resulted in increased PHL production (53, 64). Many σ^{70}-dependent promoters are regulated by transcriptional repressors that bind upstream of the transcriptional start site and impede binding of RNA polymerase. There are also examples, however, where repressors bind downstream of the transcription start site and function by interfering with promoter clearance (9). This is also the case with PhlF, which has its operator located at +140 to +169 relative to the *phlA* transcriptional start site.

Consistent with reports that downstream operators are less effective (50), repression by PhlF is not absolute. It is notable, however, that in addition to the *phO* operator, PhlF also interacts with two DNA regions closer to the transcription start site (Fig. 3 and Fig. 6). The length of these sequences (7 and 9 bp) and the fact that PhlF does not bind to oligonucleotides containing those sequences suggest these are not discrete PhlF binding sites. Rather, it is possible that PhlF bound at the *phO* operator interacts with these regions to form a more stable interaction. This suggests that binding of PhlF leads to significant structural reorganization of the *phlA* promoter, and this could also contribute to inhibition of transcription.

Some features of PhlF show similarities to those of the TetR repressor. First, the sequence and structure of the *phl* operator, *phO*, are very similar to those of the *tetO* operator located within the *tetR-tetA* intergenic region (2). Second, the putative helix-turn-helix motif present at the amino terminus of the PhlF protein (4, 12, 54) shows interesting similarities with the helix-turn-helix of TetR. Third, the active state of TetR is a homodimer (22), and we have shown in this study that PhlF forms a dimer in solution, suggesting that it may be active as a homodimer. Based on these similarities, it can be hypothesized that PhlF and TetR bind DNA in similar ways.

Mechanism of autoinduction. Many biosynthetic or degradation pathways are regulated by positive or negative feedback (5, 8, 18, 60). Recently, Schnider-Keel and colleagues showed that addition of PHL to the growth medium induced both production of PHL and expression of a *phlA'-'lacZ* gene fusion (54). Autoinduction by PHL required the *phlF* gene, but it was not determined whether PHL itself or a breakdown product was required, nor whether PhlF was directly or indirectly involved in the process. Using defined components in vitro, we established that PHL interacts directly with PhlF to reduce its binding to the *phO* operator. Furthermore, we also determined that the corepressor salicylate also acts directly via PhlF, this time to enhance its binding to *phO*. Since corepression by salicylate occurs even in the presence of high concentrations of PHL, it is speculated that autoinducer and corepressor recognize different sites on the PhlF protein.

To better characterize the PhlF interaction with the two secondary metabolites, a more detailed analysis is needed. Based on current data, however, we suggest that PHL interacts physically with PhlF and probably induces a conformational change such that the affinity of the repressor for *phO* drops, resulting in separation of the complex. Salicylate would stabilize the general structure of the repressor and therefore result in longer preservation of the complex. The concentrations of PHL and salicylate required for in vitro assays are higher than the concentrations used in the physiological study reported by Schnider-Keel et al. (54). The reason for that is not known, but it can be speculated that under physiological conditions, the PHL and the salicylate concentrations inside the cells may be significantly higher than those in the medium. Alternatively, it may be that aggregates of PhlF protein, which cannot bind DNA, are still capable of binding PHL or salicylate in the in vitro assays.

Conclusion. Biosynthesis of PHL is regulated at the transcriptional level by PhlF. The *phlA-phlF* intergenic region displays a complex organization in that *phlA* is transcribed from a σ^{70} RNA polymerase-dependent promoter that is likely to overlap the promoter of the divergently transcribed *phlF* gene. Repression by PhlF is due to its interaction with a specific sequence, *phO,* located downstream of the *phlA* promoter, suggesting that the repression may occur by inhibition of promoter clearance. This repression occurs only in the early log phase, after which the repressor is inefficient because of its interaction with the inducer, PHL. Salicylate can interact with PhlF to stabilize its interaction with the *phlA* promoter, leading to tighter repression of PHL production. Interaction of these secondary metabolites with PhlF may contribute to the complex regulation of PHL biosynthesis. Further work is required to determine how environmental signals and physiological cues combine to control PHL production.

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