Regulation of production of the antifungal metabolite 2,4-diacetylphloroglucinol in 
Pseudomonas fluorescens F113: genetic analysis of phlF as a transcriptional repressor

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INTRODUCTION

Exploiting the potential of fluorescent pseudomonads to act as crop protectants has become the focus of many research groups. Pseudomonas spp. have been investigated as potential biological control agents due to their ability to colonize the rhizosphere and protect plants against a range of agronomically important fungal diseases (Weller & Cook, 1983; Stutz et al., 1986; Loper, 1988). Their biocontrol capabilities result largely from their ability to produce a battery of antifungal metabolites. The production of the antifungal metabolite 2,4-diacetylphloroglucinol by many fluorescent 
Pseudomonas spp. has been seen to play a major role in the biocontrol of a range of plant pathogens, including Pythium ultimum, Gaeumannomyces graminis var. trici and Thielaviopsis basicola (Keel et al., 1990; Vincent et al., 1991; Fenton et al., 1992; Levy et al., 1992).

Previous work in this laboratory showed that phloroglucinol produced by Pseudomonas fluorescens F113 inhibits growth of Pythium ultimum in vitro (Shanahan et al., 1992) and protects sugar beet seedlings from damping-off disease, caused by Pythium ultimum in soil microcosms (Fenton et al., 1992).

The widespread application of pseudomonads as biocontrol agents in agriculture is impeded, however, by their variable performance under field conditions (Weller, 1988; Cook, 1993; Dowling & O’Gara, 1994). An understanding of how biocontrol bacteria regulate the expression of genes involved in the inhibition of pathogens is likely to be a prerequisite for predicting the environmental conditions for the optimum performance of these bacteria.

The antifungal metabolite 2,4-diacetylphloroglucinol plays a major role in the biocontrol capabilities of Pseudomonas fluorescens. The phloroglucinol biosynthetic locus of P. fluorescens F113 has been isolated previously. From nucleotide sequence data, a putative regulator gene (phlF) was identified upstream and divergently transcribed from the phlACBD phloroglucinol biosynthetic genes. PhlF shows similarity to various transcriptional repressors in the EMBL database and exhibits a helix–turn–helix motif in its amino acid sequence. phlF was cloned into an expression vector and the PhlF protein product was purified. Gel retardation experiments demonstrated PhlF to be a DNA-binding protein and showed that it binds to the phlA–phlF intergenic region. Introduction of phlF into P. fluorescens F113 in multiple copies resulted in repression of phloroglucinol production in this strain. This effect was mediated at the transcription level since the expression of a phloroglucinol biosynthetic gene fusion in this background was equally repressed.

Furthermore, the inactivation of phlF results in derepression of phloroglucinol production in this strain.

Keywords: biocontrol, repressor, transcriptional control, Pseudomonas fluorescens

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The genes involved in the biosynthesis of the phloroglucinol molecule have been cloned from different strains (Vincent et al., 1991; Fenton et al., 1992) and the sequence of the entire biosynthetic locus is now available for P. fluorescens strain Q2-87 in the EMBL database (accession no. U41818). Six ORFs have been identified within a 6.5 kb segment of DNA from strain Q2-87 which is sufficient to transfer phloroglucinol biosynthetic capability to recipient strains of Pseudomonas spp. that did not previously produce the antifungal metabolite (Cook et al., 1995). The genes phlA, phlC, phlB and phlD have been reported to be contained within a large transcriptional unit, previously shown to be required for phloroglucinol production (Bangera & Thomashow, 1996; Thomashaw et al., 1997). Their predicted products show similarities with proteins involved in fatty acid and polyketide biosynthesis (Cook et al., 1995; Thomashaw et al., 1997). phlE is associated with the presence of the red pigment that is usually present in media when phloroglucinol is produced (Bangera & Thomashow, 1996; Keel et al., 1996) and is hypothesized to be involved in the transport of phloroglucinol out of the cell (Thomashaw et al., 1997). However, the precise role of each gene in the biosynthetic pathway has not yet been elucidated.

The phlF gene located upstream of the biosynthetic genes shows some similarity to transcriptional repressor genes from a wide variety of organisms and is the focus of this study. In this paper we show that the phlF gene product is a DNA-binding protein which binds specifically to the phlA–phlF intergenic region. We also characterize phlF as a repressor specific for the biosynthesis of the phloroglucinol antifungal metabolite.

**METHODS**

**Media and culturing conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas* strains were grown at 28 °C in sucrose asparagine (SA) medium (Scher & Baker, 1982) and low K+ minimal medium supplemented with PAS salts (Braz et al., 1995), amended with 50 mM succinate as sole carbon source. The media were supplemented, where indicated, with 100 μM FeCl3 for high iron conditions. *Escherichia coli* strains were grown at 37 °C in (LB) broth with shaking (Sambrook et al., 1989). Antibiotics were used at the following concentrations (μg ml⁻¹) for *Pseudomonas*: tetracycline (Tc), 75; streptomycin (Sm), 100; chloramphenicol (Cm), 200; kanamycin (Km), 50; and for *E. coli*: Tc, 25; Cm, 30; Km, 25; ampicillin (Ap), 100.

**Recombinant DNA techniques.** Small- and large-scale plasmid DNA isolation was performed using QiaGen Plasmid Mini and Maxi Kits, respectively, according to the manufacturer’s specifications. Restriction digests and ligation procedures were performed as described by Sambrook et al. (1989). Chromosomal DNA was isolated according to the method described by Chen & Kuo (1993). Following electrophoretic separation, DNA fragments were purified from gels using the Qiaex II Gel Extraction Kit according to the manufacturer’s specifications (Qiagen). Plasmids were introduced into *E. coli* and *Pseudomonas* by electroporation (Farinha & Kropinski, 1990) or mobilized into *Pseudomonas* by triparental mating using the helper plasmid pRK2013 (Figurski & Helsinki, 1979). Southern blotting of 0.8% agarose gels was performed by capillary transfer onto a solid support membrane (Hybond-N; Amersham) with an alkaline 0.4 M NaOH elution buffer according to the manufacturer’s instructions. Probe labelling, hybridization and detection were carried out using the chemiluminescent DIG-High Prime DNA Labelling and Detection Kit II according to the protocols of the manufacturer (Boehringer Mannheim). Oligonucleotide primers were synthesized commercially.

Deregulated biosynthetic clones were constructed as follows. The phloroglucinol biosynthetic region of pCU203 was cloned as a 6 kb BamHI fragment into the medium-copy-number broad-host-range vector pBRR1MCs. A 1 kb SacI deletion generated pRSc-8 containing a subclone of the biosynthetic region in which phlE was deleted. An 8.3 kb EcoRI DNA fragment containing the biosynthetic locus, including phlB but a truncated and inactive phlF gene, was cloned into the medium-copy-number vector pMPP220, generating pMPE8.3. The mobile phloroglucinol biosynthetic gene fusions carried by the plasmids pCU106 and pCU107 were constructed by cloning a BamHI–Spel fragment and an EcoRI–Spel fragment of the phloroglucinol biosynthetic locus of F113 from pCU203 into pMPP220 such that the phlD gene forms a transcriptional gene fusion with the promoterless lacZ gene of pMPP220 (Fig. 1). phlF is truncated and inactive in pCU107 but intact and functional in pCU106. A phlA::lacZ transcriptional fusion pCU102 was constructed by cloning a 1.8 kb KpnI fragment from pBSL-8 into pMPP220, such that the phlA gene is fused to lacZ. phlF is truncated in this construct. The cloning of the 1.8 kb SalI–KpnI fragment from pCU203 into pMP190 generated the phlF transcriptional fusion pCU109. The entire phlF ORF was subcloned on a 2.3 kb SalI–BamHI fragment from pCU203 into a broad-host-range vector, pBRR1MCs, generating pBSL-8.

phlF mutants were created by cloning the internal SacI–KpnI fragment of phlF into narrow-host-range vector pK18. A resulting pKREG1 suicide construct was introduced into F113 by electroporation and the integrants were selected by their resistance to Km. The resulting phlF mutant (F113-phlF), in which the pK18 plasmid had integrated into the chromosomal phlF copy, was verified by Southern blot hybridization.

**Determination of nucleotide sequence and sequence analysis.** A series of subclones of the phlF locus were constructed (Table 1) and the nucleotide sequence was determined by primer walking using an Applied Biosystems 370 automated sequencer. The sequence data were assembled using DNASTAR software package (DNASTAR, Madison, WI, USA) and analysed using BLAST (Altschul et al., 1990) at the National Centre for Biotechnology Information (http://www3.ncbi.nlm.nih.gov/). DNASTAR software was employed for multiple sequence alignments.

**Construction of PhlF expression plasmid.** phlF was amplified by PCR using pBSL-8 as the DNA template and the oligonucleotide primers employed were HTD1 (5′-TGTGAT-CCATGGCCGCTAAACC-3′) and HTID3 (5′-ACCC- GAAAGTCTGGCTCCGGCG-3′). The restriction enzyme sites for NcoI and BgIII, which were incorporated into the amplified DNA fragment by the primers, are indicated by single- and double-underlining, respectively. The NcoI site is incorporated at the putative ATG translational start site of phlF and the BgIII site is positioned in the correct reading frame in the 5′ orientation with respect to the putative stop codon which is modified and hence removed. The PCR product encompassing phlF was restricted with NcoI and BgIII, isolated and purified, and subsequently cloned into the NcoI-BgIII sites of the expression vector pQE-60 of the
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. fluorescens</strong></td>
<td>Wild-type Phl** HCN** Prt** Lac**</td>
<td>Shanhah et al. (1992)</td>
</tr>
<tr>
<td>F113</td>
<td>pflF** HCN** Prt** Km**</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>Na** Str** Rif** lac** ara** gal** mtl** F** recA** ura**</td>
<td>Gottesman et al. (1981)</td>
</tr>
<tr>
<td>SG13009(pREP4)</td>
<td>pK18 Cloning vector, narrow host range, ColE1</td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>pBSL-8 2 kb–lacZα fragment from pCU203 in pMP190, Cm**</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pK18</td>
<td>Cloning vector, broad host range, IncP IncQ</td>
<td></td>
</tr>
<tr>
<td>pK18</td>
<td>pBSL-8 Cloning vector, narrow host range, ColE1</td>
<td></td>
</tr>
<tr>
<td>pMP190</td>
<td>Promoterless lacZ vector, IncP Cm** Sm**</td>
<td>Spanik et al. (1987)</td>
</tr>
<tr>
<td>pMP220</td>
<td>Promoterless lacZ vector, IncP Tc**</td>
<td>Spanik et al. (1987)</td>
</tr>
<tr>
<td>pQE-60</td>
<td>Expression plasmid, Ap**</td>
<td>Qiagen, Inc.</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid, Tr** Mob** ColE1 Km**</td>
<td></td>
</tr>
<tr>
<td>pBF01-8</td>
<td>6 kb BamHI fragment carrying phloroglucinol biosynthetic region in pBBR1MCS, Cm**</td>
<td>This study</td>
</tr>
<tr>
<td>pBSc-8</td>
<td>4.9 kb BamHI–SacI fragment from pCU203 in pBBR1MCS, Cm**</td>
<td>This study</td>
</tr>
<tr>
<td>pCU102</td>
<td>4.8 kb KpnI fragment from pBSc-8 in pMP190, Cm**</td>
<td>This study</td>
</tr>
<tr>
<td>pCU106</td>
<td>4.8 kb KpnI fragment from pBSc-8 in pMP190, Cm**</td>
<td>This study</td>
</tr>
<tr>
<td>pCU107</td>
<td>5.4 kb BamHI–SphI fragment from pCU203 in pMP220, pflF** Tc**</td>
<td>This study</td>
</tr>
<tr>
<td>pCU109</td>
<td>5.4 kb BamHI–SphI fragment from pCU203 in pMP220, pflF** Tc**</td>
<td>This study</td>
</tr>
<tr>
<td>pCU203</td>
<td>6 kb BamHI fragment carrying phloroglucinol biosynthetic region in pSUP106, Cm**</td>
<td>This study</td>
</tr>
<tr>
<td>pKREG1</td>
<td>400 bp SacI–KpnI fragment from pCU203 in pK18, Km**</td>
<td>This study</td>
</tr>
<tr>
<td>pME8.3</td>
<td>8.3 kb EcoRI fragment containing entire phloroglucinol biosynthetic locus with truncated pflF gene in pMP220, Tc**</td>
<td>This study</td>
</tr>
<tr>
<td>pBSL-8</td>
<td>2.3 kb BamHI–SacI fragment from pCU203 containing pflF in pBBR1MCS, Cm**</td>
<td>This study</td>
</tr>
<tr>
<td>pQE-60.27</td>
<td>700 bp NcoI–BglII fragment containing PCR-amplified pflF in pQE-60</td>
<td>This study</td>
</tr>
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</table>

QIAexpress system (Qiagen), generating the recombinant plasmid pQE-60.27 (Table 1). This construct places six consecutive histidine residues (6 x His-tag) at the C-terminus of the PhlF protein sequence without altering the folding of the resulting protein. Ni-NTA resin has specific affinity for proteins with an affinity tag of six histidine residues, allowing the one-step purification of PhlF with six histidine residues.

Transformant colonies containing the appropriate insertion were identified by restriction analysis and subsequently transformed into E. coli strain SG31009(pREPA4) (Gottesman et al., 1981). Transformant strains expressing PhlF were initially analysed by SDS-PAGE. Freshly inoculated 10 ml cultures were grown at 37°C with shaking to an OD<sub>600</sub> of 0.5 and protein overexpression was induced from the E. coli phage T5 promoter in pQE-60 upon the addition of 2 mM IPTG. After 2 h further incubation, 1 ml samples were removed and centrifuged at 14000 r.p.m. in a microcentrifuge, resuspended in 50 µl Laemmli buffer (Laemmli, 1970) and analysed by SDS-PAGE. The inserted DNA fragments from plasmids of strains which were seen to overproduce a protein of the appropriate size were sequenced to confirm the correct DNA fragment had been cloned.

Overexpression and purification of PhlF. A 1% (v/v) inoculum of an overnight culture of E. coli SG31009(pREP4)/(pQE-60.27), grown at 37°C with shaking, was added to 1 litre LB medium containing 100 µg Ap ml<sup>−1</sup> and 25 µg Km ml<sup>−1</sup> and grown at 30°C for 5–6 h. Overexpression of PhlF was induced by the addition of 0.1 mM IPTG. After 3–4 h further incubation at 30°C, the cells were harvested by centrifugation (8000 g for 10 min), washed once and re-
The phloroglucinol biosynthetic locus of F113 was determined using the Bradford assay with bovine serum albumin manufacturer’s instructions. Protein concentrations were determined using the Bradford assay with bovine serum albumin (BSA) as standard (Bradford, 1976).

**Gel retardation assays.** DNA fragments 2.9 and 10.6 were amplified using PCR with pBSc-8 as the DNA template and primers specific for the amplification of the intergenic promoter region of the biosynthetic locus; primer 2 (5′-GCTG-GGAGAAGCCGAGACAGG-3′), primer 6 (5′-ATTAT-GGCATGGGGACC-3′), primer 9 (5′-AATGGGA- TGAGAACAGG-3′), primer 10 (5′-CAAGAACGTCTAGA-TGACCCCTGA-3′) (see Fig. 4). A third DNA fragment, 7.U, was amplified as above, using primer 7 (5′-CAATAGGT-TGTTTTCGTAC-3′) and the universal forward primer (Genosys). Fragment 7.U of 280 bp was restricted with Alul which generates two DNA fragments of 180 and 100 bp; the 180 bp band was chosen and isolated. The DNA fragments were 5′-end-labelled with [32P]ATP using polynucleotide kinase (Boehringer Mannheim).

Gel retardation assays were performed as described by Ebbole & Zalkin (1989). The binding reaction was performed in a 25 μl reaction volume containing 20 mM Tris/HCl (pH 8.0), 10% (v/v) glycerol, 1 mM EDTA, 5 mM MgCl2, 200 mM KCl, 2 mM dithiothreitol, 50 μg bovine serum albumin ml−1, 50 μg calf thymus DNA ml−1, 50 μg poly[d(I-C)] ml−1, probe, end-labelled DNA fragments and cell extracts containing overexpressed PhlF and Ni-NTA-enriched PhlF. The reaction was incubated for 50 min at room temperature followed by the addition of 5 μl 50% glycerol and electrophoresis at 100 V through a native polyacrylamide gel containing 2.5% glycerol in TAE buffer (0.04 M Tris/acetate, pH 7.5, 2 mM EDTA). Following electrophoretic separation at 110 V for 3 h, the gel was dried and exposed overnight at −80 °C to X-Omat film (Kodak).

**Detection of phloroglucinol production and β-galactosidase assays.** Pseudomonas test strains were assayed for phloroglucinol production by HPLC using the method described by Shanahan et al. (1993). β-Galactosidase assays were performed by the colorimetric procedure described by Miller (1972), using two drops of chloroform and 1 drop of 0.1% SDS to permeabilize the cells. All reported enzyme activities are the means of several replicate assays of at least three independently grown cultures and are expressed in Miller units. pMP220 and pMP190 were also mobilized into each strain and the background β-galactosidase activity was measured as a control.

**Detection of HCN and protease production.** Strains to be screened were grown overnight in triplicate in 10 ml SA broth amended with 100 μM FeCl2 and 10 μl aliquots were spotted onto appropriate plates. For the HCN assay, test strains were spotted on SA agar plates amended with 100 μM FeCl2 for 24–48 h. The production of HCN by Pseudomonas strains was determined using HCN indicator paper (Castric & Castric, 1983). Production of HCN was visually recorded by the intensity of blue coloration of the indicator paper placed in the lid after 24 and 48 h growth. The protease assay was performed using 3% (w/v) skim milk agar plates. Pro tease production was measured as the diameter of the clear zones of proteolysis around protease-positive colonies after 24 and 48 h growth.

**RESULTS**

**Organization of the phloroglucinol biosynthetic locus in P. fluorescens F113**

The phloroglucinol biosynthetic locus of F113 was previously isolated by screening a genomic library by complementation of a mutant (F113G22) deficient in
phloroglucinol production (Fenton et al., 1992). The 6 kb phloroglucinol biosynthetic clone, pCU203, was sequenced and found to contain five complete ORFs and one partial ORF. Sequence analysis of the complementing plasmid, pCU203, revealed a similar arrangement of ORFs and strong identity at the nucleic and amino acid levels with the phloroglucinol locus of *P. fluorescens* Q2-87 (Fig. 1) (Bangera & Thomashow, 1996). The four biosynthetic genes *phlA*, *phlB*, *phlC*, and *phlD* are transcribed in the same direction as is a partial ORF, *orfY*, located downstream from *phlD*. *phlF*, which is located upstream from *phlA*, is transcribed divergently from the phloroglucinol biosynthetic genes. This study focuses on *phlF* and its possible role in the regulation of the phloroglucinol biosynthetic genes.

The sequence of *phlF* is similar to known repressor genes

The *phlF* gene of F113 consists of an ORF of 627 bp, with a corresponding predicted protein of 209 aa with a predicted molecular mass of 23 570 Da, which, interestingly, is 6 aa longer than the proposed *phlF* gene product of Q2-87 (Bangera & Thomashow, 1996). The deduced protein sequence was compared with those in the EMBL database by means of the BLAST program. At the amino acid sequence level, the predicted PhlF protein shows similarity to a group of transcriptional regulators, including six proteins implicated as DNA-binding transcriptional regulators (Figs. 3). The deduced protein displays the strongest similarity to a putative DNA-binding repressor protein of the rapamycin biosynthetic locus in *Streptomyces hygroscopicus*, sharing 24.5% identity with the proposed protein of orfY (Schwecke et al., 1995). The most conserved region of the amino acid sequence in relation to the repressor proteins is significantly at the N terminus (Fig. 2), which exhibits a helix-turn-helix DNA-binding motif (Brennan & Matthews, 1989). This strongly suggests that *phlF* encodes a DNA-binding protein. This region was also found to be conserved in the amino acid sequence of PhlF of *P. fluorescens* Q2-87 (Bangera & Thomashow, 1996).

Overexpression and partial purification of PhlF

To verify the predicted DNA-binding ability of the protein encoded by *phlF*, PhlF was overexpressed in *E. coli*. Expression of PhlF in host strain *E. coli* SG13009 was induced by the addition of IPTG to the medium. Two bands of overexpressed protein were detected, by using SDS-PAGE, from cell extracts of IPTG-induced *E. coli* harbouring pQE-60.27 (Fig. 3). These intense bands were not observed in cell extracts of the uninduced strain nor in cell extracts of *E. coli* containing the pQE-60 vector alone. The molecular mass of the larger band (25 000 Da) closely approximates to the predicted molecular mass of the PhlF protein (23 370 Da), suggesting that the PhlF protein was produced in *E. coli* SG13009(pREP4)(pQE-60.27) on induction with IPTG.

The sequence of the PCR fragment encompassing *phlF* which was subcloned in pQE-60.27 was verified and proved to be correct. The smaller sized band (18 000 Da) is thought to be a derivative of PhlF which is truncated at the N terminus (*PhlF*) produced by *E. coli* SG13009(pREP4)(pQE-60.27). Analysis of the supernatant fluids and inclusion body pellets of the whole-cell lysates by SDS-PAGE revealed that the majority of PhlF was insoluble. To increase the amount of PhlF expressed in the soluble fraction, cells of *E. coli* SG13009(pREP4)(pQE-60.27) were grown at 30 °C with shaking and induced with a lower concentration of IPTG at 0.1 mM. PhlF with the 6 × His-tag (see Methods) was then enriched by Ni-NTA column chromatography (Fig. 3). The lower induced band was co-purified using the Ni-NTA affinity columns, suggesting that it is the 6 × His-tag PhlF protein which has been truncated at the N-terminal domain. This may be a result of PhlF being translated from a false translational start site within the *phlF* sequence when it is expressed in the *E. coli* SG13009 host.

Specific binding of PhlF to the *phlA*-phlF intergenic region

Gel retardation assays were used to investigate the DNA-binding activity of PhlF. The *phlA*-phlF intergenic region spans 421 bp between the proposed *phlA* and *phlF* translational start sites. Three shorter overlapping
DNA fragments spanning parts of this region were amplified by PCR (Fig. 4) and used as substrates for gel retardation assays with extracts containing over-expressed PhlF (data not shown) and partially purified PhlF (Fig. 4). Samples of 0·8 ng of each of the fragments of DNA migrated freely through the polyacrylamide gels. However, when the DNA fragments were incubated with 0·5 µg aliquots of Ni-NTA-enriched PhlF extract, prior to being applied to a polyacrylamide gel, the migration of the 2.9 and 7.µ fragments was retarded.

![Fig. 3.](image)

**Fig. 3.** (a) SDS-PAGE showing overexpression of PhlF. The numbers to the left show the positions of the protein size markers. Lanes 1 and 2 contain cell extracts from, respectively, uninduced and IPTG-induced *E. coli* containing the pQE-60 vector control plasmid. Lanes 3 and 4 contain cell extracts from, respectively, uninduced and IPTG-induced *E. coli* containing the pQE-60.27 phlF expression plasmid. The arrows to the right show the positions of induced protein bands. (b) SDS-PAGE Ni-NTA affinity column enrichment of non-denatured cell-free extracts of SG13009(pREP4)(pQE-60.27) IPTG-induced cultures. Lane 1 contains cell-free extracts of the induced cultures prepared under non-denaturing conditions. The position of the bands of induced PhlF are marked with arrows to the right. Lane 2 contains the flow-through of the Ni-NTA column in which the induced bands have disappeared. Lane 3 contains the eluant from the washed column containing the enriched bands of the 6x-His-tag PhlF protein.

![Fig. 4.](image)

**Fig. 4.** (a) PCR amplification of the DNA fragments spanning the *phlA–phlF* intergenic region used in the gel retardation assays. The translational start sites of the divergently transcribed *phlA* and *phlF* genes are indicated. The large-headed arrows show the location and orientation of the primers used in the PCR amplification of the DNA fragments; the distances from the start of *phlA* are shown in parenthesis. The resultant DNA fragments are shown with their relative sizes indicated below and their allocated names are indicated on the left hand side. (b) Specificity of binding of PhlF to the DNA fragments spanning the *phlA–phlF* intergenic region. The resultant blots from the gel retardation assays are shown. For each binding reaction of gel (i), 0–8 ng labelled fragment 10.6 was used. Similarly, 0.8 ng labelled fragment 2.9 was applied to the binding reactions of gel (ii) and the binding reactions of gel (iii) contained 0·8 ng labelled fragment 7.µ. In addition, for all binding reactions 1 µg poly[d(I-C)] was used as non-specific competitor DNA. In binding reactions 2 and 3 in gels (i)–(iii), 0·5 µg enriched PhlF extract was used. To reactions 3 in all gels, a 125-fold excess of the respective unlabelled DNA fragment as specific competitor DNA was added. The positions of migration of bands of free DNA fragments and DNA–protein complexes are marked with arrows.
Table 2. Repression of phloroglucinol production and transcription of the phlD::lacZ fusion in F113 on addition of phlF subcloned in a medium-copy-number vector (pBSL-8)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phloroglucinol production [µg ml⁻¹ (OD₆₀₀ unit)⁻¹]</th>
<th>β-Galactosidase activity (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F113(pBBR1MCS)</td>
<td>50 ± 6</td>
<td>–</td>
</tr>
<tr>
<td>F113(pBSL-8)</td>
<td>&lt; 1</td>
<td>–</td>
</tr>
<tr>
<td>F113(phlD::lacZ)(pBBR1MCS)</td>
<td>–</td>
<td>1152 ± 64</td>
</tr>
<tr>
<td>F113(phlD::lacZ)(pBSL-8)</td>
<td>–</td>
<td>40 ± 40</td>
</tr>
</tbody>
</table>

Assays were performed in triplicate on late-exponential-phase cultures grown in SA medium amended with 100 µM FeCl₃ and the values represent the mean of triplicate cultures ± SEM.

but the 10.6 fragment migrated equidistant to the free DNA fragment. This suggests that PhlF had bound to the 2.9 and 7.6 fragments but not to the 10.6 DNA fragment. As this apparent binding occurred in the presence of poly[d(I-C)] non-specific competitor DNA, the binding of PhlF was shown to be specific for the fragments. In the control reactions, a 125-fold excess of unlabelled DNA fragment was added along with the PhlF extract to the respective labelled fragments during incubation. When specific competitor DNA was added in excess to the binding reactions of fragment 2.9 and 7.6, no gel shift was observed. The labelled fragments were specifically out-competed for binding to PhlF by the unlabelled fragment in excess and migrated in a similar fashion to the free DNA fragments. In contrast, gel retardation studies with cell extracts of the IPTG-induced and -uninduced E. coli cultures harbouring pQE-60 did not reveal any DNA binding with the DNA fragments described (data not shown). This experiment showed that PhlF binds specifically to the pbaA–phlF intergenic region. Binding of PhlF to fragments 2.9 and 7.6 but not to fragment 10.6 suggests that the PhlF-binding site is upstream of primer 6. The 2.9 and 7.6 DNA fragments overlap by approximately 110 bp. The observation that PhlF retarded the migration of the 2.9 and 7.6 fragments to approximately the same extent suggests that each fragment contains only one binding location. Therefore, the binding site of PhlF may be located in the overlapping region of the fragments (Fig. 4). However, the possibility that more than one binding site for PhlF exists within the pbaA–phlF intergenic region cannot be overlooked.

Repression of phloroglucinol production and transcription of the biosynthetic genes by phlF in F113

The negative role of phlF on phloroglucinol production was further investigated by the mobilization of pBSL-8 into F113 by triparental mating. The introduction of multiple copies of phlF on pBSL-8 in F113 leads to a reduction of over 90% in phloroglucinol production after 20 h growth in SA medium, supplemented with chloramphenicol (Table 2). To further investigate the repressive effect of phlF, the β-galactosidase activity of cells with pCU107, which contains the phlD::lacZ transcriptional fusion, was tested in strain F113 containing the phlF subclone and the control strain. Expression from the phlD::lacZ reporter plasmid, pCU107, was reduced by over 90% in the presence of multiple copies of phlF (Table 2). This demonstrates that transcription of the phlD biosynthetic gene is repressed by phlF in F113.

Phloroglucinol production is derepressed on mutation of phlF in F113

A phlF chromosomal mutant, F113-phlF−, was constructed as described in Methods. Southern blot hybridization verified that the pKREG1 suicide plasmid was integrated in the chromosome at the phlF locus of the F113-phlF− genome, thereby disrupting phlF (data not shown). The pattern of phloroglucinol production of F113-phlF− was compared to the wild-type over a time-course experiment (Fig. 5). Phloroglucinol production over the first 12 h of growth was significantly higher in the mutant when compared to the wild-type strain F113. After 6 h of growth phloroglucinol production is detected in the phlF mutant but not in wild-type F113. Phloroglucinol production after 12 h of growth is significantly greater in the phlF mutant with respect to the wild-type. It would appear from these results that phloroglucinol production is induced, or possibly derepressed, at an earlier stage in the phlF mutant. It is also interesting to note that the growth of F113-phlF− is slower during the early exponential phase when it produces more phloroglucinol than the wild-type. Phloroglucinol production is at a maximum in the late exponential phase and similar levels are observed for both mutant and wild-type F113.
production was over twofold higher in supernatants of the F113-\textit{phlF} culture \(0.38 \pm 0.1 \ \mu \text{g ml}^{-1} \ \text{(OD}_{600} \ \text{unit}^{-1})\) than in the wild-type F113 \(0.16 \pm 0.04 \ \mu \text{g ml}^{-1} \ \text{(OD}_{600} \ \text{unit}^{-1})\). After 20 h in low potassium succinate minimal broth, phloroglucinol production was over eightfold higher in supernatants of the F113-\textit{phlF} mutant \(1.96 \pm 0.7 \ \mu \text{g ml}^{-1} \ \text{(OD}_{600} \ \text{unit}^{-1})\) than in the wild-type \(2.3 \pm 0.3 \ \mu \text{g ml}^{-1} \ \text{(OD}_{600} \ \text{unit}^{-1})\). In both media the significantly higher levels of phloroglucinol in supernatants of the F113-\textit{phlF} mutant suggests that \textit{phlF} plays a significant repressive role in phloroglucinol production in these media.

**PhlF does not have a repressive effect on other secondary metabolite production**

The secondary metabolites HCN and protease, as well as phloroglucinol, are known to be under similar global control from a two component system in \textit{P. fluorescens} (Corbell & Loper, 1995; Dunne et al., 1996). Strain F113(pBSL-8), in which phloroglucinol production was repressed, and the F113-\textit{phlF} mutant were assessed in their production of the HCN and protease metabolites by plate assays, as described in Methods. The F113-\textit{phlF} mutant appeared identical to the wild-type in its production of HCN and protease after 24 and 48 h. Also, the F113 strain carrying multiple copies of \textit{phlF} [F113(pBSL-8)] did not differ in its production of HCN and protease after 24 and 48 h growth when compared to the control strain [F113(pBBR1MCs)]. This suggests that the increase in gene dosage of \textit{phlF} did not affect the production of HCN or protease in this strain. As such the repressive ability of \textit{phlF} appears to be specific for phloroglucinol production.

**DISCUSSION**

Genes involved in the biosynthesis of antibiotics and other polyketide metabolites show a marked tendency for clustering (for a review see Hopwood & Sherman, 1990) and adjacent genes in these clusters often represent loci-linked regulatory genes, usually upstream and divergently transcribed to the biosynthetic genes (Postle et al., 1984). This arrangement has been described in the case of the \textit{phlF} gene of the phloroglucinol biosynthetic locus (Bangera & Thomashow, 1996). Predictions based on the sequence of \textit{phlF} suggest that it encodes a DNA-binding repressor protein due to similarities between the deduced amino acid sequence of the \textit{phlF} gene product and sequences of some comparably sized proteins known to function as repressors (Fig. 2). A common feature of these repressors and the PhlF protein is the presence of a helix–turn–helix motif associated with DNA-binding ability/activity. One of the best known repressor proteins is TetR which forms a dimer and binds to two tandem nearly identical palindromic operator sequences within the intergenic region of the \textit{tetR} and \textit{tetA} genes (Hinrichs et al., 1994). The binding of the TetR protein regulates the expression of the tetracycline resistance determinant antiporter TetA at the transcriptional level. In this study, the overexpressed PhlF protein exhibited DNA-binding ability and was shown to bind specifically to the \textit{phlA–phlF} intergenic space. Therefore, the repressive activity of PhlF appears to be a direct result of the binding of the protein to operator sequences located in the intergenic region and inhibiting transcription from the promoter of the phloroglucinol biosynthetic genes. However, palindromic operator sequences were not identified in this region.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Time-course of phloroglucinol production of F113 (\textcircled{1}) and the \textit{phlF} mutant F113-\textit{phlF} (\textcircled{2}) in SA medium amended with 100 \textmu M FeCl\textsubscript{3}. Triplicate cultures of both strains were assayed every 6 h for phloroglucinol production according to the method described in Methods. The mean value of these extractions is shown in (a) with the SD represented as an error bar for each timepoint. This graph indicates that phloroglucinol production of the F113-\textit{phlF} mutant was significantly derepressed during the exponential phase. The graph in (b) shows the growth of these cultures during the experiment. The growth (measured as OD\textsubscript{600}) of each strain was sampled from triplicate cultures at each timepoint and the mean values of these readings are presented in the graph with the SD represented as an error bar for each timepoint. The growth of F113-\textit{phlF} was slightly retarded in the exponential phase during which time it showed an increase in phloroglucinol production.}
\end{figure}
During this study, we have compiled evidence which proves that phlF acts as a negative regulator of phloroglucinol production, which is responsible for repression of the phloroglucinol biosynthetic genes at the transcriptional level. High copy numbers of phlF in the wild-type background inhibits phloroglucinol production in F113 and reduces the level of transcription of the biosynthetic phlD gene fusion by over 90%. Second, a mutation of phlF leads to increased phloroglucinol production in vitro. Phloroglucinol production in the phlF mutant increased with respect to wild-type F113 levels in all three media tested, suggesting that phloroglucinol production was derepressed on inactivation of phlF. It is possible that in SA medium F113 phloroglucinol production is fully derepressed in late exponential phase and therefore does not exhibit any difference to the repressor mutant during later phases of growth.

The time-course experiment of phloroglucinol production from the phlF mutant seems to indicate that the repressive action of PhlF is time-dependent; phlF appears to play a role in preventing phloroglucinol production in the early stages of growth. High phloroglucinol production early in the growth cycle may be detrimental to the cell. It was indeed observed that growth of F113-phlF was delayed compared to that of the wild-type. Similar effects of phloroglucinol on cell growth were reported previously for the phloroglucinol-overproducing strain Q2-87 (pPHL5122) which was seen to lower cell density in broth culture and died more rapidly than the Q2-87 wild-type strain (Bonsall et al., 1997). The inhibitory mode of action of the phloroglucinol molecule has not yet been investigated and little is known about the inherent phloroglucinol resistance of the phloroglucinol-producing strains. The addition of synthesized phloroglucinol to liquid cultures of F113 in early exponential phase stops growth and replication of the culture (data not shown), indicating that the accumulation of phloroglucinol during this phase may be self-inhibitory. It also appears that production of phloroglucinol is fully derepressed at the maximum levels of induction for F113. It is tempting to speculate that PhlF may play an important role in preventing phloroglucinol accumulation early in the growth cycle, when it may be deleterious to the strain. While the evolutionary advantages of many antibiotics may be as agents of competition, some secondary metabolites with (perhaps incidental) antimicrobial activities may also have other functions, such as as a stimulator of aerial mycelium formation in Streptomyces alboniger (Kondo et al., 1988).

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