Complex regulation of AprA metalloprotease in *Pseudomonas fluorescens* M114: evidence for the involvement of iron, the ECF sigma factor, PbrA and pseudobactin M114 siderophore

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In the soil bacterium *Pseudomonas fluorescens* M114, extracellular proteolytic activity and fluorescent siderophore (pseudobactin M114) production were previously shown to be co-ordinately negatively regulated in response to environmental iron levels. An iron-starvation extracytoplasmic function sigma factor, PbrA, required for the transcription of siderophore biosynthetic genes, was also implicated in M114 protease regulation. The current study centred on the characterization and genetic regulation of the gene(s) responsible for protease production in M114. A serralyisin-type metalloprotease gene, *aprA*, was identified and found to encode the major, if not only, extracellular protease produced by this strain. The expression of *aprA* and its protein product were found to be subject to complex regulation. Transcription analysis confirmed that PbrA was required for full *aprA* transcription under low iron conditions, while the ferric uptake regulator, Fur, was implicated in *aprA* repression under high iron conditions. Interestingly, the iron regulation of AprA was dependent on culture conditions, with PbrA-independent AprA-mediated proteolytic activity observed on skim milk agar supplemented with yeast extract, when supplied with iron or purified pseudobactin M114. These effects were not observed on skim milk agar without yeast extract. PbrA-independent *aprA* expression was also observed from a truncated transcriptional fusion when grown in sucrose asparagine tryptone broth supplied with iron or purified pseudobactin M114. Thus, experimental evidence suggested that iron mediated its effects via transcriptional activation by PbrA under low iron conditions, while an as-yet-unidentified sigma factor(s) may be required for the PbrA-independent *aprA* expression and AprA proteolytic activity induced by siderophore and iron.

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INTRODUCTION

Extracellular protease production by the soil isolate *Pseudomonas fluorescens* M114 was previously shown to be co-ordinately regulated with pseudobactin siderophore biosynthesis, both being negatively regulated by iron (Adams *et al.*, 1994). The iron starvation extracytoplasmic function (ECF) alternative sigma factor, PbrA, was found to be required for full transcription of *P. fluorescens* M114 pseudobactin biosynthetic (and receptor) genes (Sexton *et al.*, 1995). PbrA was also implicated in the regulation of

protease activity as, in addition to being siderophore-negative, a *pbrA* mutant was protease-negative on skim milk agar (Sexton *et al.*, 1995). However, *P. fluorescens* M114 pseudobactin biosynthetic mutants, M1 (*pbsC*[−]) and FA6 (*pbsE*[−]), displayed wild-type levels of proteolytic activity under low iron conditions (Adams *et al.*, 1994; Sexton, 1995), demonstrating that PbrA regulation of protease was not indirect via its regulation of pseudobactin. A consensus sequence for the Fe²⁺-dependent transcription repressor protein Fur (ferric uptake regulator) was identified in the promoter region of *pbrA* and *pbrA* transcription was found to be repressed under high iron conditions (Sexton *et al.*, 1996), suggesting that iron regulation of protease may be mediated via Fur regulation of PbrA. Nevertheless, the identity of the *P. fluorescens* M114 protease(s) and the molecular basis for PbrA and iron regulation remained to be elucidated.

Previously, numerous *Pseudomonas* spp. have been shown to produce and secrete serralyisin-type metalloproteases

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Abbreviations: ECF, extracytoplasmic function; Fur, ferric uptake regulator; SAT, sucrose asparagine tryptone medium; SAT+YE, SAT supplemented with 0.15% yeast extract; SM, skim milk agar; SMYE, SM supplemented with 0.15% yeast extract.

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(EC 3.4.24.40) (Ahn *et al.*, 1999; Chabeaud *et al.*, 2001; Chessa *et al.*, 2000; Duong *et al.*, 1992; Kawai *et al.*, 1999; Kumeta *et al.*, 1999; Liao & McCallus, 1998; Woods *et al.*, 2001; GenBank accession no. AJ007827). In *P. fluorescens* B52, Woods *et al.* (2001) reported that expression of the serralyisin-type metalloprotease gene, *aprX*, was negatively regulated by iron at the transcriptional level. While the authors suggested the possible involvement of a PbrA homologue, they were unable to test this hypothesis, as they failed to isolate a *pbrA* insertion mutant of this strain. In *Pseudomonas aeruginosa*, PvdS (the ECF sigma factor exhibiting greater than 85 % identity to PbrA at the amino acid level) was found to co-ordinately regulate genes involved in the production of pyoverdine (pseudobactin-type) fluorescent siderophore, exotoxin A and the PrpL (Protease IV) serine protease gene, *prpL* (Vasil & Ochsner, 1999; Wilderman *et al.*, 2001; Ochsner *et al.*, 2002). Additionally, Shigematsu *et al.* (2001) reported that transcription of the *P. aeruginosa* alkaline (serralyisin-type) protease *aprA* gene was negatively regulated by iron and AprA levels were reduced in a *pvdS* mutant. However, while a micro-array study confirmed PvdS regulation of genes involved in the production of pyoverdine, exotoxin A and PrpL, *aprA* was not among the genes found to be PvdS-regulated (Ochsner *et al.*, 2002), although expression was regulated by iron. The possible direct involvement of Fur in the repression of *P. aeruginosa aprA* transcription has been suggested. However, while in gel shift assays addition of purified *P. aeruginosa* Fur led to a shift in the *aprA* band, the intensity of the shifted band was noted to be weak (Shigematsu *et al.*, 2001). Thus, the role of Fur and PvdS in *P. aeruginosa aprA* regulation remains to be fully elucidated. A consensus sequence termed the iron starvation (IS) box, previously identified in the promoters of iron-regulated genes (Rombel *et al.*, 1995), was shown to be required for PvdS recognition in *P. aeruginosa* (Wilson *et al.*, 2001).

Interestingly, a pyoverdine-mediated siderophore signalling system was shown to regulate the activity of PvdS and a second ECF sigma factor, FpvI, in *P. aeruginosa*. Experimental data were consistent with a model whereby these ECF sigma factors associate with a transmembrane anti-sigma factor, FpvR, and are thus rendered inactive. (Ferri-) pyoverdine binding to its outer membrane receptor, FpvA, initiates a signal cascade from FpvA, via FpvR, to PvdS and FpvI, resulting in sigma factor dissociation from FpvR and consequently sigma factor activation. Active FpvI directs the transcription of the (ferri-)pyoverdine receptor gene, *fpvA*, while active PvdS directs transcription of the pyoverdine biosynthetic genes, genes required for exotoxin A production and *prpL* (Lamont *et al.*, 2002; Shen *et al.*, 2002; Beare *et al.*, 2003; Rédly & Poole, 2003).

This study was initiated to investigate the role of iron and more specifically PbrA in the regulation of *P. fluorescens* M114 proteolytic activity. We report that this strain produces a serralyisin-type metalloprotease, AprA, which encounters complex transcriptional regulation involving

iron and PbrA. Furthermore, an unforeseen observation of PbrA-independent regulation of AprA-mediated M114 proteolytic activity under certain culture conditions was investigated.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *P. fluorescens* strains were cultured at 28 °C in sucrose asparagine (SA) medium (Scher & Baker, 1982), in sucrose asparagine tryptone medium (SAT; SA medium supplemented with 0.4 % tryptone and 1 mM CaCl₂) or in SAT supplemented with 0.15 % yeast extract (SAT+YE). For low iron conditions, glassware was washed in 6 M HCl and rinsed thoroughly with deionized water. For high iron conditions, the medium was supplemented with 100 µM FeCl₃. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37 °C. Antibiotics were added, when required, at the following concentrations: tetracycline, 25 µg ml⁻¹ for *E. coli* and 70 µg ml⁻¹ for *P. fluorescens*; chloramphenicol, 30 µg ml⁻¹ for *E. coli* and 300 µg ml⁻¹ for *P. fluorescens*; kanamycin, 50 µg ml⁻¹ for *E. coli* and 25 µg ml⁻¹ for *P. fluorescens*; spectinomycin, 50 µg ml⁻¹ for *P. fluorescens*; and ampicillin, 50 µg ml⁻¹ for *E. coli*.

Assessment of proteolytic activity. Proteolytic activity was qualitatively assessed by streaking test strains onto skim milk agar (SM) plates (10 % skim milk, 1.5 % purified agar) or SM plates supplemented with 0.15 % yeast extract (designated SMYE). Yeast extract (L21) was manufactured by Oxoid. Following 72 h incubation at 28 °C, the diameters of the zones of clearing were compared. For high iron conditions, the agar was supplemented with 100 µM FeCl₃. Where indicated, agar was supplemented with 50 µM purified pseudobactin M114.

To assess the ability of various strains to induce proteolytic activity in strains with a protease-negative phenotype, test strains were cross-streaked horizontally and close to vertically streaked protease-negative strains. Alternatively, cell-free supernatant from the test strains grown in SA medium was added to wells cut into SM, with protease-negative strains streaked in close proximity. Following 72 h incubation at 28 °C, the protease phenotype of the initially protease-negative strains was assessed.

Extracellular proteolytic activity in cell-free supernatant (cultures grown in SAT or SAT+YE broth) was quantitatively assessed using the azocasein assay, as previously described (Sarath *et al.*, 1989) with some modifications. Briefly, 2 % azocasein in 50 mM Tris/HCl buffer, pH 8, was used as a substrate and supernatant was incubated with substrate at 28 °C for 18 h. OD₄₄₀ readings correlating with extracellular proteolytic activity were divided by OD₆₀₀ readings to correct for cell density differences. A Beckman DU 640 spectrophotometer was used for all OD measurements. One unit was defined as that amount of enzyme required to produce a change of 0.01 in OD₄₄₀/OD₆₀₀.

Purification of pseudobactin M114. Pseudobactin M114 was purified from *P. fluorescens* M114 cell-free supernatant (culture grown in Casamino acids medium for 24 h) using C18 chromatography as described by Baysse *et al.* (2002). The concentration of pseudobactin was estimated spectrophotometrically at 400 nm using the extinction coefficient, ϵ_{400} , of $2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Meyer & Abdallah, 1978).

DNA manipulations. Restriction enzyme digests, ligation of DNA fragments, transformation of *E. coli* and agarose gel electrophoresis were performed as described by Sambrook *et al.* (1989). Plasmid DNA was isolated using the Qiaprep spin miniprep kit (Qiagen). Chromosomal DNA was isolated by the method of Chen & Kuo

Table 1. Bacterial strains, plasmids and primers used in this study

Strain or plasmid	Relevant properties*	Source or reference	
Strains			
<i>P. fluorescens</i>			
M114	Wild-type, Ap ^r Fluorescence ⁺ Protease ⁺	O’Gara <i>et al.</i> (1986)	
FA10	M114 <i>pbrA</i> mutant, Gm ^r Sp ^r Ap ^r Fluorescence [−] Protease [−]	Sexton <i>et al.</i> (1995)	
M1	M114 <i>pbsC</i> mutant, Fluorescence [−] Km ^r	Adams <i>et al.</i> (1994)	
FA6	M114 <i>pbsE</i> mutant, Fluorescence [−] Sp ^r	Sexton (1995)	
FA15	M114 <i>aprA</i> mutant, Protease [−] , Km ^r	This study	
FA16	M114 <i>pbrA aprA</i> double mutant, Fluorescence [−] Protease [−] , Gm ^r Sp ^r Ap ^r Km ^r	This study	
F113	Wild-type, Fluorescence ⁺	Shanahan <i>et al.</i> (1992)	
CHA0	Wild-type, Fluorescence ⁺	Voisard <i>et al.</i> (1994)	
MT3A	Wild-type, Fluorescence ⁺	National University of Ireland – Cork Culture Collection	
<i>P. putida</i> WCS358	Wild-type, Fluorescence ⁺	Geels & Schippers (1983)	
<i>Pseudomonas</i> sp. B10	Wild-type, Fluorescence ⁺	Moores <i>et al.</i> (1984)	
<i>Pseudomonas</i> strains x330, x338, x522, x531 and x159	All wild-type, Fluorescence ⁺	Moënne-Loccoz <i>et al.</i> (2001)	
<i>E. coli</i>			
DH5α	Cloning and subcloning host, ϕ80 <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>) U169 <i>hsdR17 recA1 endA1 thi-1</i>	Sambrook <i>et al.</i> (1989)	
TOP10	Cloning and subcloning host	Invitrogen	
HB101	<i>recA hsdR hsdM strA pro leu thi</i>	Boyer & Roulland-Dussoix (1969)	
Plasmids			
pRK2013	Helper plasmid, IncP Tra ⁺ Mob ⁺ ColE1, Km ^r	Figurski & Helinski (1979)	
pCR2.1-TOPO	Cloning vector, <i>lacZ</i> α fragment containing MCS, fl origin, ColE1, Km ^r Ap ^r	Invitrogen	
pBBR1MCS	Cloning vector, broad host-range, Cm ^r	Kovach <i>et al.</i> (1994)	
pK18mob	Mobilizable suicide cloning vector, Km ^r	Schafer <i>et al.</i> (1994)	
pCUB23	1·98 kb fragment containing <i>aprA</i> from <i>P. fluorescens</i> M114 in pBBR1MCS, Cm ^r	This study	
pMP220	Promoterless <i>lacZ</i> vector, IncP, Tc ^r	Spaink <i>et al.</i> (1987)	
pCUB15	M114 <i>aprA-lacZ</i> transcriptional fusion in pMP220 (502 bp <i>aprA</i> promoter fragment) Tc ^r	This study	
pCUB16	M114 <i>aprA-lacZ</i> transcriptional fusion in pMP220 (442 bp <i>aprA</i> truncated promoter fragment) Tc ^r	This study	
pCUB17	M114 <i>aprA-lacZ</i> transcriptional fusion in pMP220 (316 bp <i>aprA</i> truncated promoter fragment) Tc ^r	This study	
pCUB20	550 bp fragment containing an internal region of <i>aprA</i> from <i>P. fluorescens</i> M114 in pK18mob, Km ^r	This study	
Primer	Sequence (5’–3’)	Primer	Sequence (5’–3’)
ALKPRTF	GTGGACGAAAGATTTCAAGTT	PSTR	ACTGTCGGCTCATGCTGAG
ALKPRTR	GTCCTGGCCGCCGCTGTAGTT	2PSTF	TCGATGAAGATGGCGAAG
APRT2F	CAGACGTGGCCAACGTGA	2PSTR	TAACCGATATAGGGACCGA
APRT2R	CGAGAACACCAGCTTGTC	3PSTF	AGTGCTGGGTGTGCTGGGTGCTTC
MAPOUT1F	CAGCGTTTCGCCTATCTGC	MP1F	CTAATCAGCCAGGCCATACATA
MAPOUT1R	CAATGAAGGCGTAAGAAAC	MP2F	CAGCGTTTAAAGGGTTGA
ENDF	CCAACACTGGCCGTGATTTTC	2BMR	CGCCATTGCCTTGTAGGTA
ENDR	CATTCCCGCGTTGTAGTCA	MPR	GGCTGAAGCTATTGATCTGGTTGT
2ENDF	AACAGTGTGGCCAACGAACCTCA	KNOF	GGCAAAAGCTCCGCAACTAAAACA
PSTF	GCGCTGATCATGATTTTCAG		

*Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sp, spectinomycin; Tc, tetracycline.

(1993). DNA fragments were purified from agarose gels using the Qiaquick gel extraction kit (Qiagen). Plasmids were conjugated into *Pseudomonas* via triparental mating using the helper plasmid pRK2013 (Figurski & Helinski, 1979). Southern analysis was performed using standard procedures (Sambrook *et al.*, 1989). Probe labelling, hybridization and detection were performed using the Enhanced Chemiluminescence ECL system (Amersham Pharmacia Biotech). PCR was routinely performed with Master Mix (Qiagen) and 20–50 pmol of each primer. Isolated genomic DNA (60 ng) or bacterial colonies, preheated at 94 °C for 10 min, provided template DNA. Primers used in this study were commercially synthesized by Sigma-Genosys and are listed in Table 1. PCR products were purified where necessary using the QIAquick PCR purification kit (Qiagen).

Cloning of the metalloprotease gene, *aprA*, from *P. fluorescens* M114. The nucleotide sequence of serralyisin-type metalloprotease genes from various strains of *Pseudomonas* were aligned. Two different pairs of protease primers were designed based on conserved regions: set 1, ALKPRTF and ALKPRTT; set 2, APRT2F and APRT2R (Table 1). The primer sets were designed such that they would result in the amplification of overlapping sequences. PCR reaction mixes were supplemented with bovine serum albumin (BSA) at a final concentration of 0.13 mg ml⁻¹. PCR amplification was performed with a *P. fluorescens* M114 colony under the following conditions for both sets of primers: 94 °C for 10 min for 1 cycle; 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min for 30 cycles; 72 °C for 5 min for 1 cycle. PCR amplification resulted in products of 547 and 550 bp with primer sets 1 and 2, respectively. Nucleotide sequence analysis revealed that the two PCR products overlapped, giving a 973 bp sequence.

Cloning the *aprA* promoter. The promoter region of *aprA* was cloned using an inverse PCR approach (Fig. 1). Sequence analysis of *aprA* revealed a unique *Xho*II site 530 bp downstream of the putative start codon of *aprA*. Genomic DNA from M114 was digested with *Xho*II and run on a 1 % TAE agarose gel. Southern blot analysis,

using the primer set 1 amplified 547 bp *aprA* PCR product as probe, was performed to identify a *Xho*II fragment containing part of the known *aprA* sequence plus the unknown upstream sequence. Size-matched fragments were gel-extracted from a second M114 *Xho*II digest and ligated with T4 DNA ligase in a dilute final volume of 500 µl to promote intramolecular ligations. Following heat inactivation of the ligase enzyme, ligated DNA was ethanol-precipitated and resuspended in PCR mix. PCR amplification with divergent primers MAPOUT1R and MAPOUT1F (Table 1), located within the *aprA* sequence upstream of the *Xho*II site, was performed under the following conditions: 94 °C for 2 min for 1 cycle; 94 °C for 1 min, 52.3 °C for 1 min, 72 °C for 4 min for 30 cycles; 72 °C for 5 min for 1 cycle. The resulting 433 bp inverse PCR product was gel-extracted and sequenced.

Cloning the 3' end of the *aprA* gene and genes downstream of *aprA*. Inverse PCR, as previously described, was used to obtain the remainder of the *aprA* coding sequence and genes downstream of *aprA* (Fig. 1). Initially, inverse PCR amplification using divergent primers ENDR and ENDF (Table 1), located within the *aprA* sequence downstream of the unique *Xho*II site, was performed using ligated *Xho*II-digested M114 DNA under the following conditions: 94 °C for 2 min for 1 cycle; 94 °C for 1 min, 55 °C for 1 min, 72 °C for 4 min for 30 cycles; 72 °C for 5 min for 1 cycle. The resulting 476 bp inverse PCR product was gel-extracted and sequenced.

The remainder of the *aprA* sequence and the sequences of downstream genes were obtained by inverse PCR amplification with ligated *Pst*I-digested M114 DNA using divergent primers ENDR and 2ENDF, located within the *aprA* sequence downstream of the unique *Pst*I site, under the following conditions: 94 °C for 2 min for 1 cycle; 94 °C for 1 min, 52 °C for 1 min, 72 °C for 4 min for 30 cycles; 72 °C for 5 min for 1 cycle. The resulting 3388 bp inverse PCR product was gel-extracted and sequenced.

Nucleotide sequencing and sequence analysis. For nucleotide sequencing, PCR products were routinely cloned in the pCR2.1-TOPO

Fig. 1. Inverse PCR strategy used to clone and sequence upstream and downstream from the initially incomplete *aprA* PCR fragment. —, Initially known sequence; - - - - - , upstream sequence obtained using inverse PCR 1; ······, downstream sequence obtained using inverse PCR 2; - · - · - ·, downstream sequence obtained using inverse PCR 3.

vector (Invitrogen). The nucleotide sequence was determined using M13 forward and reverse primers on an Applied Biosystems PRISM 310 Automated Genetic Analyser. The complete sequence of the 3388 bp inverse PCR product was obtained by primer walking using the primers PSTF, PSTR, 2PSTF, 2PSTR and 3PSTF (Table 1). The sequence data were assembled using the DNASTAR software package. The GenBank database was searched using the National Centre for Biotechnology Information with BLAST algorithms (Altschul *et al.*, 1997). The *P. fluorescens* Pf0-1 genome sequencing project (Joint Genome Institute Microbial Sequencing Program; www.jgi.doe.gov) was also used for BLAST searches. DNASTAR software was employed for multiple sequence alignments.

Construction of *aprA* knockout mutant. An *aprA* knockout mutant, FA15, was constructed by a single crossover of the suicide vector pK18mob containing an internal *aprA* fragment. A 550 bp internal *aprA* fragment, amplified with primers APRT2F and APRT2R, was cloned, via pCR2.1-TOPO, into the multicloning site of the kanamycin-resistant vector pK18mob, resulting in pCUB20. pCUB20 was transformed into *E. coli* DH5 α and subsequently conjugated into *P. fluorescens* M114. Kanamycin-resistant transconjugants, resulting from a single crossover event, were screened on SM. Disruption of *aprA* was confirmed by PCR with the *aprA*-specific primer, KNOF, and M13 forward primer (M13 sites flank the pK18mob multicloning site) under the following conditions: 94 °C for 10 min for 1 cycle; 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min for 30 cycles; 72 °C for 5 min for 1 cycle. A PCR product of about 1.2 kb was obtained with FA15 due to *aprA* disruption, while as expected no PCR product was obtained with M114.

Construction of *pbrA aprA* double knockout mutant. To construct FA16, a *pbrA aprA* double knockout mutant, pCUB20, was conjugated from DH5 α into FA10, the spectinomycin-resistant *pbrA* mutant. Spectinomycin- and kanamycin-resistant transconjugants, resulting from a single crossover event, were screened on SMYE. Disruption of *aprA* in FA16 was also confirmed by PCR as described for FA15.

Complementation of *aprA* mutants. To complement the *aprA* mutants, the entire *aprA* gene and upstream promoter (405 bp upstream of the putative translational start site) was PCR-amplified from M114 genomic DNA (600 ng) using the Expand High Fidelity PCR System (Roche) with primers MP1F and 2BMR (Table 1) under the following cycle extension conditions: 94 °C for 2 min for 1 cycle; 94 °C for 1 min, 59 °C for 1 min, 72 °C for 2 min for 10 cycles; 94 °C for 1 min, 59 °C for 1 min, 72 °C for 2 min + 5 s per cycle for 20 cycles; 72 °C for 7 min for 1 cycle. The resulting 1.98 kb PCR product was gel-extracted and cloned, via pCR2.1-TOPO, into the multicloning site of pBBR1MCS, downstream of and in the same orientation as the pBBR1MCS *lac* promoter, resulting in pCUB23. pCUB23 was transformed into DH5 α and subsequently conjugated into *P. fluorescens* FA15 and FA16. The proteolytic activity of the resulting transconjugants was assessed on SM and SMYE under low and high iron conditions.

Construction of *aprA-lacZ* transcriptional fusions. Three *aprA* transcriptional fusions of different lengths were constructed in the reporter plasmid pMP220 via initial cloning of PCR-amplified *aprA* promoter fragments in the pCR2.1-TOPO vector (Invitrogen). The promoter fragments were cut out of correctly oriented TOPO clones using the *Asp*718 and *Xba*I sites flanking the PCR insert site on pCR2.1-TOPO and cloned directly into the multicloning site of pMP220, upstream of the promoterless *lacZ* gene. pCUB15, containing a 502 bp fragment, including 405 bp upstream of the *aprA* putative translational start site, was amplified from M114 genomic DNA using the primers MP1F and MPR under the following conditions: 94 °C for 2 min for 1 cycle; 94 °C for 1 min, 51.4 °C for 1 min, 72 °C for 1 min for 30 cycles; 72 °C for 4 min for 1 cycle.

pCUB16, containing a 442 bp PCR product, including 345 bp upstream of the *aprA* putative translational start site, was amplified using the primers MP2F and MPR under the following conditions: 94 °C for 2 min for 1 cycle; 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min for 30 cycles; 72 °C for 4 min for 1 cycle. pCUB17 contained a 316 bp PCR product, including 219 bp upstream of the *aprA* putative translational start site, and was amplified using the primers KNOF and MPR under the following conditions: 94 °C for 2 min for 1 cycle; 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min for 30 cycles; 72 °C for 4 min for 1 cycle.

β -Galactosidase assays. β -Galactosidase activity was measured as described by Miller (1972). All assays were done in at least triplicate. Miller units were corrected for background values obtained with the *lacZ* fusion vector pMP220 without insert.

Primer extension. Total RNA was isolated from 7×10^9 cells of M114(pCUB17), grown in SAT under low and high iron conditions for 16 h, using the RNeasy total RNA kit (Qiagen). Following DNase treatment, phenol/chloroform extraction and ethanol precipitation, RNA quantity and quality were assessed using $A_{260/280}$. RNA integrity was also checked on a 1% TBE agarose gel. Primer extension was performed as described by Pujic *et al.* (1998) with some modifications: 100 pmol primer MPR, which annealed to the sequence between 74 and 97 nt downstream of the putative ATG translational start site, was labelled with γ - 32 P using T4 polynucleotide kinase. RNA and labelled primer were hybridized at 65 °C for 10 min followed by incubation at 50 °C for 1.5 h. Reverse transcription was performed with avian myeloblastosis virus reverse transcriptase at 42 °C for 1.5 h. The T7 sequencing kit (USB) was employed for reference sequence reactions using primer MPR with pCUB23, containing M114 *aprA* as template.

RESULTS

Genetic characterization of the *P. fluorescens* M114 metalloprotease gene, *aprA*

To study the molecular regulation of *P. fluorescens* M114 proteolytic activity, it was necessary to identify, clone and sequence the M114 protease gene(s). Previously, numerous *Pseudomonas* spp. have been shown to produce and secrete serralyisin-type metalloproteases (EC 3.4.24.40) (Ahn *et al.*, 1999; Chabaud *et al.*, 2001; Chessa *et al.*, 2000; Duong *et al.*, 1992; Kawai *et al.*, 1999; Kumeta *et al.*, 1999; Liao & McCallus, 1998; Woods *et al.*, 2001; GenBank accession no. AJ007827). Addition of the metalloprotease inhibitor EDTA to M114 supernatant led to a significant reduction in M114 proteolytic activity (data not shown), strongly suggesting that M114 also produced and secreted a metalloprotease. An internal fragment of a metalloprotease gene was cloned from M114 using primers with sequence homology to conserved regions of metalloprotease genes from different strains of *Pseudomonas* spp. Inverse PCR was used to obtain the remainder of the *aprA* coding sequence, promoter and downstream genes. Sequence alignment revealed that the M114 metalloprotease gene, designated *aprA*, displayed the highest percentage identity (90% at the DNA level; 93% at the deduced amino acid level) to *P. fluorescens* Pf0-1 *aprA* (*P. fluorescens* Pf0-1 genome sequencing project; www.jgi.doe.gov), with greater than 75% identity at the amino acid level to serralyisin-type metalloproteases of other *P. fluorescens* strains, *Pseudomonas tolaasii* and

Pseudomonas brassicacearum and 54 % identity to AprA from *P. aeruginosa*.

Genes encoding a putative serralyisin inhibitor, designated *aprI*, a putative ATP-binding cassette (ABC) protein, designated *aprD*, and part of a putative membrane fusion protein, designated *aprE*, were identified downstream of *aprA*. Thus, M114 *aprA* formed part of a gene cluster conserved in *Pseudomonas* spp. (Ahn *et al.*, 1999; Chabeaud *et al.*, 2001; Kawai *et al.*, 1999; Liao & McCallus, 1998).

Altered response of *P. fluorescens* M114 proteolytic activity to environmental iron levels on SMYE

Previously it has been shown that *P. fluorescens* M114 proteolytic activity, as measured by a zone of clearing on SM, is reduced when the medium is supplemented with iron (100 µM ferric chloride) (Adams *et al.*, 1994; Sexton *et al.*, 1995). Interestingly, in this study we demonstrated that, when grown on SMYE, M114 exhibited an altered response to iron levels whereby the addition of a range of FeCl₃ concentrations (10, 30, 70, 100 µM) did not significantly reduce proteolytic activity (Table 2; Fig. 2a and b). In contrast, 10 µM FeCl₃ was sufficient to reduce M114 proteolytic activity on SM without yeast extract (Table 2).

The M114 *pbrA* mutant, FA10, previously shown to be completely protease-negative on SM, under both low and high iron conditions (Sexton *et al.*, 1995), was also protease-negative when grown on SMYE without added iron (Table 2; Fig. 2c). However, on SMYE supplemented with 100 µM FeCl₃, FA10 exhibited a low but significant level of PbrA-independent proteolytic activity (Table 2; Fig. 2d). In fact, 30 µM FeCl₃ was sufficient to induce PbrA-independent proteolytic activity in FA10 on SMYE and the level of activity increased with increasing iron concentrations (Table 2). Thus, under certain growth conditions, M114 was capable

of producing PbrA-independent, but iron-dependent, protease activity.

Siderophore induction of PbrA-independent protease activity

On further investigating PbrA-independent protease activity, it was noted that streaking the *pbrA* mutant, FA10, in close proximity to wild-type M114 on low iron SMYE conferred on FA10 the ability to produce low level protease activity (Table 2; Fig. 2e). This was demonstrated as a zone of clearing around the FA10 streak in closest proximity to the wild-type streak. Interestingly, this effect was not seen on SM without yeast extract.

Subsequently, when the ability of *P. fluorescens* M114 cell-free supernatant to induce protease activity in FA10 was assessed, it was noted that only supernatant from M114 grown under low iron conditions was capable of doing so. This suggested that under certain growth conditions, wild-type *P. fluorescens* M114 produced an iron-regulated signal molecule capable of inducing PbrA-independent protease activity in FA10. Interestingly, two different pseudobactin M114 biosynthetic mutants, M1 and FA6, failed to restore protease activity, suggesting that pseudobactin M114 siderophore itself was acting as a signal molecule. This hypothesis was confirmed when protease activity was observed in FA10 when grown on SMYE supplemented with purified pseudobactin M114 (Fig. 2f).

The ability of various wild-type fluorescent *Pseudomonas* spp. (*Pseudomonas* x330, x338, x522, x531, x159, *Pseudomonas putida* WCS358, *P. fluorescens* F113, *P. fluorescens* CHA0, *Pseudomonas* sp. B10 and *P. fluorescens* MT3A) to induce protease production in FA10 was also assessed and only *Pseudomonas* sp. B10 could do so. Pseudobactin B10 is structurally identical to pseudobactin M114 (Ruangviriyachai, 1991) and *P. fluorescens* M114 has been

Table 2. Proteolytic activity from *P. fluorescens* M114 and the *pbrA* mutant, FA10, and analysis and complementation of *aprA* mutant, FA15, and the *pbrA aprA* double mutant, FA16, on SM and on SMYE

+, Protease-positive, colony surrounded by zone of clearing (the number of + symbols indicates the sizes of the zones of clearing relative to each other); –, protease-negative, colony not surrounded by zone of clearing; ±, colony surrounded by an opaque zone indicating very low but reproducible levels of proteolytic activity; (+), colony surrounded by an opaque zone indicating low levels of proteolytic activity; NA, not applicable; ND, not determined.

Added FeCl ₃ (µM)	SM					SMYE					SMYE + sid*
	0	10	30	70	100	0	10	30	70	100	
M114	++++	+	+	+	+	++++	+++	+++	+++	+++	NA
FA10	–	–	–	–	–	–	–	±	(+)	+	+
FA15	–	ND	ND	ND	–	–	ND	ND	ND	–	–
FA15(pCUB23)	++++	ND	ND	ND	++++	++++	ND	ND	ND	++++	NA
FA16	–	ND	ND	ND	–	–	ND	ND	ND	–	–
FA16(pCUB23)	–	ND	ND	ND	++++	–	ND	ND	ND	++++	++

*Cross-streaked with *P. fluorescens* M114 or supplied with purified pseudobactin M114 siderophore.

Fig. 2. Assessment of proteolytic activity of wild-type *P. fluorescens* M114 and the *pbrA* mutant, FA10, on 10% SMYE. (a) M114 under low iron conditions (0 μM FeCl_3 added); (b) M114 under high iron conditions (100 μM FeCl_3 added); (c) FA10 under low iron conditions; (d) FA10 under high iron conditions; (e) FA10 (vertical streak) with M114 (horizontal streak) streaked in close proximity under low iron conditions; (f) FA10 under low iron conditions supplemented with 50 μM pseudobactin M114.

shown to take up pseudobactin B10 through PbuA, the pseudobactin M114 outer-membrane receptor (Morris *et al.*, 1992). Thus, siderophore induction of protease in FA10 was only observed with pseudobactin M114 type siderophores.

PbrA-dependent and -independent proteolytic activity in *P. fluorescens* M114 was mediated by AprA

To determine if *aprA* was responsible for the casein protease activity detected on SM and on SMYE, an *aprA* knockout mutant, termed FA15, was constructed. FA15 exhibited a protease-negative phenotype under all growth conditions tested and cross-streaking with M114 wild-type failed to induce protease activity in the mutant (Table 2). Complementation of the mutant with the entire *aprA* gene cloned in pCUB23 restored full protease activity on SM.

A *pbrA aprA* double mutant, termed FA16, was also constructed and, in contrast to FA10, this double mutant was protease-negative on SMYE under high iron conditions (Table 2) and pseudobactin M114 could not induce proteolytic activity on SMYE under low iron conditions

(Table 2). Complementation with the intact *aprA* gene cloned in pCUB23 restored proteolytic activity under these conditions (Table 2). Thus, the PbrA-independent proteolytic activity seen in FA10 on SMYE under high iron conditions or when supplied with M114 siderophore under low iron conditions was demonstrated to be mediated by AprA. Interestingly, when FA15 was complemented with pCUB23, constitutive proteolytic activity was observed, consistent with *aprA* expression being driven by the *lacZ* promoter situated upstream of its own promoter in pCUB23. However, in the FA16 background constitutive proteolytic activity from pCUB23 was not observed. It appears that in the absence of *pbrA*, the *lacZ* promoter in pCUB23 was not active under low iron conditions.

Role of iron and PbrA in *aprA* transcriptional regulation

To investigate the transcriptional regulation of M114 *aprA*, an *aprA-lacZ* transcriptional fusion (pCUB15) was constructed (Fig. 3a, b) and the role of iron and PbrA in *aprA* expression was studied. Initially, however, it was necessary to identify a low iron broth medium that would induce

Fig. 3. (a) Nucleotide sequence of the region upstream of *P. fluorescens* M114 *aprA* numbered relative to the transcriptional start site. The transcriptional start site (A) is underlined and in bold. A methionine (M) residue is positioned over the putative start codon. Two overlapping putative Fur boxes are in bold and underlined with a solid line and with a dashed line, respectively. Sequences bearing similarity to the IS consensus sequence are boxed with dotted lines. The putative PAD box is in bold and surrounded by a dashed box. The region implicated in negative transcriptional regulation is underlined with dots. This region contains an inverted repeat in bold italics, with the complementary nucleotides marked + and -, and a sequence showing homology to the negative regulatory region of *Erwinia carotovora* subsp. *carotovora* (renamed *Pectobacterium carotovorum* subsp. *carotovorum*) *priW* promoter (Marits *et al.*, 2002) is boxed. Double overlines indicate the positions of forward primers MP1F, MP2F and KNOF, used in the construction of transcriptional fusions pCUB15, pCUB16 and pCUB17, respectively. (b) *aprA-lacZ* transcriptional fusion pCUB15 (314 bp upstream of the subsequently identified transcriptional start site) and truncated fusions: pCUB16 (254 bp upstream of the transcriptional start site) and pCUB17 (128 bp upstream of the transcriptional start site) with the relative positions of the putative Fur/IS region (white) and the putative PAD box (black) marked. The arrow represents the transcriptional start site.

M114 proteolytic activity. When grown in SAT (for 16 h), M114 proteolytic activity, as measured using the azocasein assay, was in agreement with results obtained with the SM assay whereby in low iron, proteolytic activity was induced and the addition of 100 μ M FeCl₃ significantly reduced this activity (low iron, 11.63 ± 0.44 units; high iron, 1.27 ± 0.4 units). However, in contrast to the protease-negative phenotype of the *pbrA* mutant, FA10, on SM, low levels of PbrA-independent proteolytic activity were detected when the mutant was grown in SAT minus added iron (low iron, 2.2 ± 0.65 units; high iron, 0.5 ± 0.6 units). Differences in the sensitivities of the two proteolytic assays may account for this discrepancy. Analysis of *aprA* transcription from pCUB15 in wild-type M114 in SAT confirmed that the addition of extracellular iron repressed *aprA* expression at the transcriptional level (Fig. 4a, b). In addition, *aprA* transcription was significantly reduced in the *pbrA* mutant, FA10, compared to wild-type, under low iron conditions, demonstrating that PbrA was required for full *aprA* transcription in SAT (Fig. 4c).

To determine if the addition of yeast extract to broth cultures resulted in the apparent abolition of iron regulation of *P. fluorescens* M114 *AprA* production, as seen on SMYE, proteolytic activity and expression from the *aprA-lacZ* transcriptional fusion, pCUB15, was quantified in SAT and in SAT + YE (Table 3). Proteolytic activity and *aprA-lacZ* expression were quantified using the azocasein assay and the Miller assay, respectively. While both M114 proteolytic activity and *aprA* expression were reduced when grown in SAT + YE compared to SAT, iron regulation of both was still observed in SAT + YE supplemented with 100 μ M FeCl₃.

Deletion analysis of the *aprA* promoter

To elucidate the mechanism of iron/PbrA regulation observed in SAT, the *aprA* promoter region was examined for the presence of consensus sequences previously implicated in iron regulation (Fig. 3a, b). Two overlapping sequences bearing significant homology (79 and 73 % identity, respectively) to the Fur box consensus sequence

Fig. 4. Expression of the long, medium and short *aprA-lacZ* transcriptional fusions, pCUB15, pCUB16 and pCUB17, respectively, conjugated in (a) wild-type M114 grown under low iron conditions (SAT-Fe), (b) M114 under high iron (+Fe) conditions (SAT+100 μ M Fe), (c) the *pbrA* mutant, FA10, under low iron conditions, (d) FA10 under high iron (+Fe) conditions and (e) FA10 under low iron conditions supplemented with 50 μ M pseudobactin M114 (+sid). β -Galactosidase activity was measured after 16 h growth and expressed in Miller units. Miller units were corrected for background values obtained with the *lacZ* fusion vector pMP220 without an insert. Background values were as follows: M114(pMP220), 10 Miller units; M114(pMP220)+Fe, 11 Miller units; FA10(pMP220), 66 Miller units; FA10(pMP220)+Fe, 8 Miller units; FA10(pMP220)+sid, 10 Miller units. Error bars represent the standard deviation between triplicate cultures.

a sequence bearing limited homology (58 % identity) to the previously suggested PbrA-dependent PAD consensus sequence (GAACTGANNCT; Sexton *et al.*, 1996) was identified between -110 bp and -122 bp relative to the putative start codon.

To investigate the role of these putative consensus sequences, truncated *aprA-lacZ* transcriptional fusions, pCUB16 and pCUB17, were constructed (Fig. 3a, b) and their activity assessed in wild-type and mutant backgrounds in SAT (Fig. 4). pCUB16 contained 345 bp upstream of the putative *aprA* start codon, including the putative Fur and IS box(es). In pCUB17, the upstream sequence, including the putative Fur/IS box(es) region, was deleted, leaving 219 bp upstream of the putative start codon. The expression of pCUB16 and pCUB17 was assessed in the M114 wild-type background and compared with the expression from the full-length fusion, pCUB15. Similarly to pCUB15, under high iron conditions, the expression from pCUB16 was significantly reduced. In contrast, however, the addition of iron did not repress expression from pCUB17 (Fig. 4b). This abolition of iron regulation seen with pCUB17 was consistent with the deletion of Fur box(es), suggesting that Fur may directly regulate *aprA*. Interestingly, under low iron conditions, expression from the two truncated fusions was significantly higher than that from the full-length fusion (Fig. 4a), suggesting that the truncated fusions lacked some type of negative regulatory site occurring between -405 bp and -345 bp relative to the putative start codon (Fig. 3a).

To further investigate the mechanism and extent of PbrA regulation of *aprA* transcription, expression from pCUB16 and pCUB17 was compared with pCUB15 expression in the *pbrA* mutant background, FA10. Under low iron conditions, expression from both truncated fusions was reduced to levels comparable with pCUB15 expression in FA10 (Fig. 4c). Thus, the region required for PbrA-mediated activation of *aprA* transcription lies within -219 bp upstream of the putative start codon, suggesting that the putative IS sequences, identified further upstream, are not required for PbrA-dependent transcription. Under

[NAT(A/T)ATNAT(A/T)ATNAT(A/T)ATN; Escobar *et al.*, 1999] were identified, positioned from -250 bp to -232 bp and from -257 bp to -239 bp relative to the putative *aprA* translational start codon. A sequence, TTAAT-N15-CGT, bearing significant homology to the *P. aeruginosa* PvdS-dependent IS consensus (TAAAT-N16-CGT; Visca *et al.*, 2002) was found overlapping the Fur boxes. Furthermore, this region also contained a second sequence, TAAAT-N16-ATT, identical to part of the IS consensus. Interestingly,

Table 3. *P. fluorescens* M114 proteolytic activity and expression from the *aprA-lacZ* transcriptional fusion, pCUB15, when cultured in SAT and SAT+YE for 20 h

Data are shown as means of three replicate samples $\pm$ SD. Miller units were corrected for background values obtained with *lacZ* fusion vector pMP220 without insert. Background values were as follows: M114(pMP220) in SAT, 14 Miller units; M114(pMP220) in SAT+Fe, 10 Miller units; M114(pMP220) in SAT+YE, 12 Miller units; M114(pMP220) in SAT+YE+Fe, 5 Miller units.

Strain	Medium	Proteolytic activity (units)	
		Low iron (0 μ M FeCl ₃)	High iron (100 μ M FeCl ₃)
M114	SAT	9.42 $\pm$ 0.81	1.3 $\pm$ 0.27
M114	SAT+YE	4.02 $\pm$ 0.39	1.37 $\pm$ 0.09
β -Galactosidase activity (Miller units)			
		Low iron (0 μ M FeCl ₃)	High iron (100 μ M FeCl ₃)
M114(pCUB15)	SAT	856 $\pm$ 94	108 $\pm$ 11
M114(pCUB15)	SAT+YE	537 $\pm$ 113	167 $\pm$ 23

high iron conditions, expression from the three promoter fusions was not comparable (Fig. 4d); expression from pCUB17 was significantly higher, indicating a significant level of iron-dependent, PbrA-independent expression from this truncated fusion.

In light of the pseudobactin induction of PbrA-independent proteolytic activity in FA10 on SMYE, the effect of added purified pseudobactin on the expression from the three *aprA-lacZ* fusions was assessed in the FA10 background under low iron conditions (Fig. 4e). Purified pseudobactin failed to stimulate expression from pCUB15 and pCUB16, but, interestingly, induced high levels of PbrA-independent expression from pCUB17.

To further investigate this phenomenon, expression of the *lacZ* gene in pCUB15 and pCUB17 was assessed in the siderophore biosynthetic mutants, M1 and FA6 (Fig. 5). Expression from the full-length fusion, pCUB15, in M1 and FA6 was comparable with expression in wild-type M114. Interestingly, pCUB17 expression in the siderophore mutants was as high or even higher than expression in

wild-type M114. Therefore, the reduced pCUB15 and pCUB17 expression observed in FA10 under low iron conditions was not an indirect effect due to the lack of pseudobactin and supports the evidence that PbrA was required for this expression. Also, PbrA-independent expression from pCUB17 under high iron conditions was iron-dependent, but not dependent on the availability of siderophore.

Determination of the *aprA* transcriptional start site

In light of the PbrA-dependent and -independent transcriptional regulation revealed by deletion analysis of the *aprA* promoter, primer extension using RNA from M114 (pCUB17) was carried out to map the *aprA* transcriptional start sites in response to low and high iron conditions. The transcriptional start site was found to be the same under both conditions (Fig. 6) and mapped 91 bp upstream of the putative translational start site. This is at the same position as the *P. fluorescens* B52 and the *P. fluorescens* LS107d2 metalloprotease gene transcriptional start sites (Burger *et al.*, 2000; Woods *et al.*, 2001). It is noteworthy that the primer

Fig. 5. Expression of the *aprA-lacZ* transcriptional fusions, pCUB15 and pCUB17, conjugated in the siderophore biosynthetic mutants M1 and FA6, and grown for 16 h in SAT under low and high (+Fe) iron conditions. β -Galactosidase activity (in Miller units) was corrected for the following background values: M1(pMP220), 21 Miller units; M1(pMP220)+Fe, 8 Miller units; FA6(pMP220), 15 Miller units; FA6(pMP220) +Fe, 19 Miller units. Error bars represent the standard deviation between triplicate cultures.

Fig. 6. Mapping the low and high iron transcriptional start sites of *aprA*. Primer extension was performed with 50 µg total RNA isolated from M114(pCUB17) grown under low iron (–Fe) and high iron (+Fe) conditions, as described in Methods. Extension products were resolved by denaturing PAGE and autoradiography. A, T, G and C represent the sequencing products obtained with MPR, the same primer used for primer extension. The transcriptional start site is indicated as +1.

extension product obtained under low iron conditions was weaker than that observed under high iron conditions. Although transcription from *aprA*–*lacZ* in pCUB17 under these two conditions was shown to be PbrA-dependent and -independent, respectively, no other primer extension products were observed under either condition.

DISCUSSION

In this study, a serralyisin-type metalloprotease gene, *aprA*, was identified in *P. fluorescens* M114, along with a downstream protease inhibitor gene, *aprI*, and genes encoding components of an ABC protease transporter, *aprD* and *aprE* (incomplete sequence). This *apr* gene organization in M114 is similar to that in other *Pseudomonas* spp. (Liao & McCallus, 1998; Ahn *et al.*, 1999; Kawai *et al.*, 1999; Kumeta *et al.*, 1999; Chessa *et al.*, 2000; Chabeaud *et al.*, 2001; Woods *et al.*, 2001; GenBank accession no. AJ007827). In common with other serralyisin-type metalloproteases, M114 AprA is most probably secreted by a Type I secretion system involving AprD, AprE and an as yet uncharacterized outer-membrane protein, AprF. Characterization and complementation of an *aprA* mutant phenotype suggested that AprA is the major, if not only, extracellular protease produced by M114. Previously, both iron and the iron starvation ECF alternative sigma factor, PbrA, were implicated

in the regulation of M114 protease activity (Sexton *et al.*, 1995), but the identity of the M114 protease and the molecular mechanism involved in its regulation by iron and PbrA remained to be elucidated. The present study revealed that *aprA* is negatively regulated by iron at the transcriptional level and that PbrA is required for full transcription under low iron conditions. Two putative binding sites for the Fur repressor protein (Fur boxes) with overlapping putative IS sequences (the *P. aeruginosa* recognition site for PvdS activation) were identified in the *aprA* promoter, in a region centred at –148 bp relative to the subsequently identified transcriptional start site. Both Fur and IS consensus sequences are usually located closer to the transcriptional start sites of target genes, typically around the –10, –35 bp region. It is difficult, therefore, to rationalize how Fur could directly influence *aprA* transcription. Nevertheless, the abolition of iron regulation in the truncated fusion (pCUB17) lacking these sequences suggested that high iron repression of M114 *aprA* transcription may be mediated directly by Fur. It is noteworthy that the possible involvement of Fur in *P. aeruginosa aprA* regulation was suggested by Shigematsu *et al.* (2001). In gel shift assays, addition of purified *P. aeruginosa* Fur led to a shift in the *aprA* band; however, the intensity of the shifted band was weak. The *pbrA* promoter also contains Fur consensus sequences and is itself transcriptionally iron-regulated. Thus, iron repression of *aprA* transcription may be additionally mediated indirectly via PbrA. The involvement of Fur in both *P. aeruginosa* and *P. fluorescens* M114 *aprA* regulation thus warrants further investigation.

In *P. aeruginosa*, while *aprA* transcription was previously shown to be iron-regulated (Shigematsu *et al.*, 2001; Ochsner *et al.*, 2002) and AprA levels were reported to be reduced in a *pvdS*[–] mutant (Shigematsu *et al.*, 2001), *pvdS* was not found to be required for *aprA* transcription (Ochsner *et al.*, 2002). In the present study it is demonstrated that PbrA is required for full *P. fluorescens* M114 *aprA* transcription under low iron conditions. However, it is clear that the putative IS consensus sequences are not required for this regulation, as PbrA-mediated regulation of *aprA* expression was still observed from the pCUB17 truncated fusion (Fig. 4). In fact, considering the observed PbrA-dependent expression from pCUB17, a potential PbrA essential sequence would have to be positioned within 128 bp of the *aprA* transcriptional start site. A sequence (CGGCAGATTTC) bearing some homology (58% identity) to a previously suggested PbrA recognition ‘PAD’ consensus sequence (GAAGTGANNNCT; Sexton *et al.*, 1996) was identified in the *aprA* promoter positioned at –19 bp to –30 bp relative to the *aprA* transcriptional start site. Interestingly, the position of the transcriptional start site supports the hypothesis that this may be the target for PbrA regulation. While further research will be necessary to confirm the PbrA recognition site on the *aprA* promoter, evidence presented here suggests that, in spite of the strong homology between PbrA and PvdS, they may recognize different target sequences.

Although this study provides evidence that *aprA* transcription is regulated by PbrA and iron, the interesting phenomenon of PbrA-independent proteolytic activity observed on the semi-quantitative SM plate assays when supplemented with yeast extract (SMYE) plus iron suggested that *aprA* regulation was more complex than previously believed. This was compounded by the fact that pseudobactin M114 appeared to act as a signal molecule inducing PbrA-independent proteolytic activity in FA10 on SMYE without added iron. These results suggest that under certain growth conditions, the addition of iron or siderophore could induce PbrA-independent protease activity in M114. Yeast extract is a complex nutrient source containing both iron and haem, but the component(s) responsible for mediating this PbrA-independent AprA activity remains to be established. Addition of yeast extract to SAT (SAT + YE) did not lead to the same pattern of proteolytic activity as that seen on SMYE. Therefore, the phenomenon cannot be explained by yeast extract containing an additional source of iron or indeed by the presence of an iron chelator. It is possible that the altered regulation may be a feature of cells growing on solid medium, rather than as planktonic cells, and may warrant further investigation. Interestingly, culture medium effects on pseudomonad gene expression have been reported by others: Yarwood *et al.* (2005) found that expression of many quorum-sensing-regulated genes in *P. aeruginosa* is delayed by complex medium components, including yeast extract.

Interestingly, transcription from the truncated fusion, pCUB17, under high iron conditions in SAT was also PbrA-independent. Furthermore, the addition of purified pseudobactin M114 to FA10 resulted in an even greater level of PbrA-independent transcription from pCUB17 than was observed under high iron conditions. Thus, expression from pCUB17 in SAT mirrored AprA proteolytic activity on SMYE. It is tempting to speculate that SMYE may represent or mimic an *in vivo* situation where the intact *aprA* promoter functions like the truncated fusion. For example, certain environmental conditions may induce a putative iron-dependent sigma factor to activate *aprA* expression independently of Fur and PbrA, thus uncoupling protease and siderophore expression.

Given the ability of iron to induce PbrA-independent *aprA* expression from pCUB17, it is also tempting to speculate that an accumulation of iron released from ferric pseudobactin may be responsible for the observed PbrA-independent *aprA* expression from pCUB17 when supplied with purified pseudobactin and consistent with a putative second sigma factor activated by iron. However, the fact that pseudobactin induced higher levels of pCUB17 expression in the FA10 background than iron alone suggests that pseudobactin itself may also influence gene expression. It was also interesting that siderophore did not induce expression from the longer *lacZ* reporter fusions, pCUB15 and pCUB16, suggesting that iron and siderophore induce expression from the second uncharacterized sigma factor only. The possible involvement

of a siderophore signalling system in the regulation of genes required for siderophore production and uptake in *P. fluorescens* M114 was previously suggested whereby the expression of pseudobactin M114 biosynthetic genes and the pseudobactin M114 receptor gene, *pbuA*, were reduced in siderophore mutant backgrounds, while expression was increased by the addition of purified pseudobactin M114 (Callanan *et al.*, 1996). However, a system similar to the *P. aeruginosa* pyoverdine siderophore signalling system (Lamont *et al.*, 2002; Shen *et al.*, 2002; Beare *et al.*, 2003; Rdly & Poole, 2003), leading to activation of *P. fluorescens* M114 PbrA, would not explain this pseudobactin M114 induction of AprA activity in the *pbrA* mutant, FA10. Nevertheless, the possibility that an as-yet-unidentified sigma factor(s) may be activated by a pseudobactin M114 siderophore cascade remains to be investigated.

Interestingly, primer extension analysis revealed the same *aprA* transcriptional start site from M114(pCUB17) grown under both low and high iron conditions (Fig. 6) and located at the same position as the *P. fluorescens* B52 and the *P. fluorescens* LS107d2 metalloprotease gene transcriptional start sites (Burger *et al.*, 2000; Woods *et al.*, 2001). Although the intensity of the primer extension band under low iron conditions was consistently weaker, this suggests that PbrA and an unidentified sigma factor may recognize the same promoter to initiate PbrA-dependent and -independent transcription, respectively. In *E. coli*, many promoters recognized by the primary sigma factor, σ^{70} , are also recognized by the stationary-phase sigma factor, σ^{32} . This regulon overlap has also been reported for *Bacillus subtilis* ECF sigma factors σ^X and σ^W , and for *Mycobacterium tuberculosis* ECF sigma factors σ^E and σ^H (reviewed by Helmann, 2002).

It is noteworthy that expression analysis of the full (pCUB15) versus truncated (pCUB16 and pCUB17) *aprA-lacZ* transcriptional fusions also revealed that there is a possible negative regulatory site in the *aprA* promoter upstream of the putative Fur binding site(s) between -314 bp and -254 bp relative to the transcriptional start site (Fig. 3a). Examination of this region failed to identify any known negative regulator binding sites, but a potential stem-loop structure was present (Fig. 3a). Interestingly, a putative negative regulatory region was also identified in the promoter of the *Erwinia carotovora* subsp. *carotovora* (renamed *Pectobacterium carotovorum* subsp. *carotovorum*) serralyisin-type protease gene, *prrW*, between -371 and -245 bp relative to the start codon (Marits *et al.*, 2002). Alignment of these two sites from *prrW* and *aprA* revealed a homologous region showing 83 % identity over 12 bp [*aprA* 5' region, -393 (-302) GCCATACATAAA (-291) -382; *prrW* 5' region, -322 GCCATTAATAAA -311; both numbered relative to translational start sites, with numbering relative to M114 *aprA* transcriptional start site in parentheses]. Site-directed mutagenesis would be required to determine if these sequences play a role in *aprA* transcriptional regulation.

In conclusion, proteolytic activity in *P. fluorescens* M114 has been shown to be mediated by a serralyisin-type metalloprotease, AprA, which is subject to complex regulation. PbrA is required for *aprA* transcription under low iron conditions, resulting in the co-ordinate production of siderophore and protease. Results also suggested a role for Fur in *aprA* repression under high iron conditions. While iron regulation of serralyisin-type metalloprotease transcription was previously reported in *P. fluorescens* B52 (Woods *et al.*, 2001), this, to our knowledge, is the first time that the iron starvation alternative sigma factor, PbrA, has been shown to be required for full transcription from a serralyisin-type metalloprotease gene of a *P. fluorescens* strain. Furthermore, PbrA-independent, but iron- or siderophore-dependent, AprA proteolytic activity and *aprA* transcription were observed on SMYE and with a truncated *aprA-lacZ* fusion, respectively, suggesting the involvement of a possible second sigma factor in *aprA* transcription. Further research, possibly involving site-directed and random mutagenesis respectively, will be required to confirm the target site for PbrA binding and for identification of the sigma factor(s) involved in PbrA-independent *aprA* expression induced by iron and siderophore.

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REFERENCES

- Adams, C., Dowling, D. N., O'Sullivan, D. J. & O'Gara, F. (1994). Isolation of a gene (*pbsC*) required for siderophore biosynthesis in fluorescent *Pseudomonas* sp. strain M114. *Mol Gen Genet* **243**, 515–524.
- Ahn, J. H., Pan, J. G. & Rhee, J. S. (1999). Identification of the *tliDEF* ABC transporter specific for lipase in *Pseudomonas fluorescens* SIK W1. *J Bacteriol* **181**, 1847–1852.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. L. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Bayse, C., Budzikiewicz, H., Fernández, D. U. & Cornelis, P. (2002). Impaired maturation of the siderophore pyoverdine chromophore in *Pseudomonas fluorescens* ATCC 17400 deficient for the cytochrome *c* biogenesis protein CcmC. *FEBS Lett* **523**, 23–28.
- Beare, P. A., For, R. J., Martin, L. W. & Lamont, I. L. (2003). Siderophore-mediated cell signalling in *Pseudomonas aeruginosa*: divergent pathways regulate virulence factor production and siderophore receptor synthesis. *Mol Microbiol* **47**, 195–207.
- Boyer, H. W. & Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* **41**, 459–472.
- Burger, M., Woods, R. G., McCarthy, C. & Beacham, I. R. (2000). Temperature regulation of protease in *Pseudomonas fluorescens* LS107d2 by an ECF sigma factor and a transmembrane activator. *Microbiology* **146**, 3149–3155.
- Callanan, M., Sexton, R., Dowling, D. N. & O'Gara, F. (1996). Regulation of the iron uptake genes in *Pseudomonas fluorescens* M114 by pseudobactin M114: the *pbrA* sigma factor does not mediate the siderophore regulatory response. *FEMS Microbiol Lett* **144**, 61–66.
- Chabeaud, P., de Groot, A., Bitter, W., Tommassen, J., Heulin, T. & Achouak, W. (2001). Phase-variable expression of an operon encoding extracellular alkaline protease, a serine protease homolog, and lipase in *Pseudomonas brassicacearum*. *J Bacteriol* **183**, 2117–2120.
- Chen, W. P. & Kuo, T. T. (1993). A simple and rapid method for the preparation of gram-negative bacterial genomic DNA. *Nucleic Acids Res* **21**, 2260.
- Chessa, J. P., Petrescu, I., Bentahir, M., van Beeumen, J. & Gerday, C. (2000). Purification, physico-chemical characterization and sequence of a heat labile alkaline metalloprotease isolated from a psychrophilic *Pseudomonas* species. *Biochim Biophys Acta* **1479**, 265–274.
- Duong, F., Lazdunski, A., Cami, B. & Murgier, M. (1992). Sequence of a cluster of genes controlling synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationships to other secretory pathways. *Gene* **121**, 47–54.
- Escobar, L., Pérez-Martin, J. & de Lorenzo, V. (1999). Opening the iron box: transcriptional metalloregulation by the Fur protein. *J Bacteriol* **181**, 6223–6229.
- Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. *Proc Natl Acad Sci U S A* **76**, 1648–1652.
- Geels, F. P. & Schippers, B. (1983). Reduction in yield depressions in high frequency potato cropping soil after seed tuber treatments with antagonistic fluorescent *Pseudomonas* spp. *Phytopathol Z* **108**, 207–214.
- Helmann, J. D. (2002). The extracytoplasmic function (ECF) sigma factors. *Adv Microb Physiol* **46**, 47–110.
- Kawai, E., Idei, A., Kumura, H., Shimazaki, K., Akatsuka, H. & Omori, K. (1999). The ABC-exporter genes involved in the lipase secretion are clustered with the genes for lipase, alkaline protease, and serine protease homologues in *Pseudomonas fluorescens* no. 33. *Biochim Biophys Acta* **1446**, 377–382.
- Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M. I. & Peterson, K. M. (1994). pBBR1MCS: a broad-host-range cloning vector. *Biotechniques* **16**, 800–802.
- Kumeta, H., Hoshino, T., Goda, T., Okayama, T., Shimada, T., Ohgiya, S., Matsuyama, H. & Ishizaki, K. (1999). Identification of a member of the serralyisin family isolated from the psychrotrophic bacterium, *Pseudomonas fluorescens* 114. *Biosci Biotechnol Biochem* **63**, 1165–1170.
- Lamont, I. L., Beare, P. A., Ochsner, U., Vasil, A. I. & Vasil, M. L. (2002). Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **99**, 7072–7077.
- Liao, C. H. & McCallus, D. E. (1998). Biochemical and genetic characterisation of an extracellular protease from *Pseudomonas fluorescens* CY091. *Appl Environ Microbiol* **64**, 914–921.
- Marits, R., Tshuikina, M., Pirhonen, M., Laasik, E. & Mäe, A. (2002). Regulation of the expression of *prtW::gusA* fusions in *Erwinia carotovora* subsp. *carotovora*. *Microbiology* **148**, 835–842.
- Meyer, J. M. & Abdallah, M. A. (1978). The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physico-chemical properties. *J Gen Microbiol* **107**, 319–328.

- Miller, J. H. (1972). *Experiments in Molecular Biology*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Moënné-Loccoz, Y., Tichy, H. V., O'Donnell, A., Simon, R. & O'Gara, F. (2001). Impact of 2,4-diacetylphloroglucinol-producing biocontrol strain *Pseudomonas fluorescens* F113 on intraspecific diversity of resident culturable fluorescent pseudomonads associated with the roots of field-grown sugar beet seedlings. *Appl Environ Microbiol* **67**, 3418–3425.
- Moore, J. C., Magazin, M., Ditta, G. S. & Leong, J. (1984). Cloning of genes involved in biosynthesis of pseudobactin, a high affinity iron transport agent of a plant growth-promoting *Pseudomonas* strain. *J Bacteriol* **157**, 53–58.
- Morris, J., O'Sullivan, D. J., Koster, M., Leong, J., Weisbeek, P. J. & O'Gara, F. (1992). Characterization of fluorescent siderophore-mediated iron uptake in *Pseudomonas* sp. strain M114: evidence for the existence of an additional ferric siderophore receptor. *Appl Environ Microbiol* **58**, 630–635.
- Ochsner, U. A., Wilderman, P. J., Vasil, A. I. & Vasil, M. L. (2002). GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. *Mol Microbiol* **45**, 1277–1287.
- O'Gara, F., Treacy, P., O'Sullivan, D., O'Sullivan, M. & Higgins, P. (1986). Biological control of phytopathogens by *Pseudomonas* spp. genetic aspects of siderophore production and root colonization. In *Iron, Siderophores and Plant Diseases*, pp. 331–339. Edited by T. R. Swinburne. New York: Plenum.
- Pujic, P., Dervyn, R., Sorokin, A. & Ehrlich, S. D. (1998). The *kdgRKAT* operon of *Bacillus subtilis*: detection of the transcript and regulation by the *kdgR* and *ccpA* genes. *Microbiology* **144**, 3111–3118.
- Rédly, G. A. & Poole, K. (2003). Pyoverdine-mediated regulation of FpvA synthesis in *Pseudomonas aeruginosa*: involvement of a probable extracytoplasmic-function sigma factor, FpvI. *J Bacteriol* **185**, 1261–1265.
- Rombel, I. T., McMorran, B. J. & Lamont, I. L. (1995). Identification of a DNA sequence motif required for expression of iron-regulated genes in pseudomonads. *Mol Gen Genet* **246**, 519–528.
- Ruangviriyachai, C. (1991). *Liquid chromatographic and complexation analysis of natural and synthetic chelating agents*. PhD thesis, University College Cork, Cork, Ireland.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sarath, G., de la Motte, R. & Wagner, F. W. (1989). Protease assay methods. In *Proteolytic Enzymes: a Practical Approach*, pp. 25–55. Edited by R. J. Beynon & J. S. Bond. Oxford: Oxford University Press.
- Schafer, A., Tauch, A., Jager, W., Kalinowski, J. & Puhler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**, 69–73.
- Scher, F. M. & Baker, R. (1982). Effects of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium* wilt pathogens. *Phytopathology* **72**, 1567–1573.
- Sexton, J. R. (1995). *Cloning and characterisation of transcriptional activator(s) required for iron regulated gene expression in Pseudomonas fluorescens M114*. PhD thesis, University College Cork, Cork, Ireland.
- Sexton, R., Gill, P. R., Jr, Callanan, M. J., O'Sullivan, D. J., Dowling, D. N. & O'Gara, F. (1995). Iron responsive gene expression in *Pseudomonas fluorescens* M114: cloning and characterization of a transcription-activating factor, PbrA. *Mol Microbiol* **15**, 297–306.
- Sexton, R., Gill, P. R., Jr, Dowling, D. N. & O'Gara, F. (1996). Transcriptional regulation of the iron-responsive sigma factor gene *pbrA*. *Mol Gen Genet* **250**, 50–58.
- Shanahan, P., O'Sullivan, D. J., Simpson, P., Glennon, J. D. & O'Gara, F. (1992). Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. *Appl Environ Microbiol* **58**, 353–358.
- Shen, J., Meldrum, A. & Poole, K. (2002). FpvA receptor involvement in pyoverdine biosynthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **184**, 3268–3275.
- Shigematsu, T., Fukushima, J., Oyama, M., Tsuda, M., Kawamoto, S. & Okuda, K. (2001). Iron-mediated regulation of alkaline proteinase production in *Pseudomonas aeruginosa*. *Microbiol Immunol* **45**, 579–590.
- Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E. & Lugtenberg, B. J. J. (1987). Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1J1. *Plant Mol Biol* **9**, 27–39.
- Vasil, M. L. & Ochsner, U. A. (1999). The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. *Mol Microbiol* **34**, 399–413.
- Visca, P., Leoni, L., Wilson, M. J. & Lamont, I. L. (2002). Iron transport and regulation, cell signalling and genomics: lessons from *Escherichia coli* and *Pseudomonas*. *Mol Microbiol* **45**, 1177–1190.
- Voisard, C., Bull, C., Keel, C., Laville, J., Maurhofer, M., Schnider, U., Défago, G. & Haas, D. (1994). Biocontrol of root diseases by *Pseudomonas fluorescens* CHA0: current concepts and experimental approaches. In *Molecular Ecology of Rhizosphere Microorganisms*, pp. 67–89. Edited by F. O'Gara, D. N. Dowling & B. Boesten. Weinheim, Germany: VCH Publishers.
- Wilderman, P. J., Vasil, A. I., Johnson, Z., Wilson, M. J., Cunliffe, H. E., Lamont, I. L. & Vasil, M. L. (2001). Characterization of an endo-protease (PrpL) encoded by a PvdS-regulated gene in *Pseudomonas aeruginosa*. *Infect Immun* **69**, 5385–5394.
- Wilson, M. J., McMorran, B. J. & Lamont, I. L. (2001). Analysis of promoters recognized by PvdS, an extracytoplasmic-function sigma factor protein from *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 2151–2155.
- Woods, R. G., Burger, M., Beven, C. A. & Beacham, I. R. (2001). The *aprX-lipA* operon of *Pseudomonas fluorescens* B52: a molecular analysis of metalloprotease and lipase production. *Microbiology* **147**, 345–354.
- Yarwood, J. M., Volper, E. M. & Greenberg, E. P. (2005). Delays in *Pseudomonas aeruginosa* quorum-sensing are conditional. *Proc Natl Acad Sci U S A* **102**, 9008–9013.