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A new regulator of pathogenicity (*bvlR*) is required for full virulence and tight microcolony formation in *Pseudomonas aeruginosa*

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LysR-type transcriptional regulators (LTTRs) are the most common family of transcriptional regulators found in the opportunistic pathogen *Pseudomonas aeruginosa*. They are known to regulate a wide variety of virulence determinants and have emerged recently as positive global regulators of pathogenicity in a broad spectrum of important bacterial pathogens. However, in spite of their key role in modulating expression of key virulence determinants underpinning pathogenic traits associated with the process of infection, surprisingly few are found to be transcriptionally altered by contact with host cells. BvlR (PA14_26880) an LTTR of previously unknown function, has been shown to be induced in response to host cell contact, and was therefore investigated for its potential role in virulence. BvlR expression was found to play a pivotal role in the regulation of acute virulence determinants such as type III secretion system and exotoxin A production. BvlR also played a key role in *P. aeruginosa* pathogenicity within the *Caenorhabditis elegans* acute model of infection. Loss of BvlR led to an inability to form tight microcolonies, a key step in biofilm formation in the cystic fibrosis lung, although surface attachment was increased. Unusually for LTTRs, BvlR was shown to exert its influence through the transcriptional repression of many genes, including the virulence-associated *cupA* and *alg* genes. This highlights the importance of BvlR as a new virulence regulator in *P. aeruginosa* with a central role in modulating key events in the pathogen–host interactome.

INTRODUCTION

Cystic fibrosis (CF) is the most prevalent autosomal recessive disorder in the Caucasian population. The leading cause of morbidity among CF patients is loss of lung function due to chronic bacterial infection (Koch & Hoiby, 1993). While a range of different bacteria establish infections in the CF lung throughout infancy, many of these are often treated successfully with antibiotics (Harrison, 2007). *Pseudomonas aeruginosa*, however, is capable of avoiding eradication, and becomes the dominant species of bacteria in the lungs from the late teens onwards. These chronic infections result in *P. aeruginosa* being the leading cause of morbidity and mortality among CF patients (Lyczak *et al.*, 2002). To establish a chronic lung infection, *P. aeruginosa* utilizes a wide range of virulence determinants, the production of which it controls through interconnection of global and local regulatory networks.

LysR-type transcriptional regulators (LTTRs) are the most common transcriptional regulators found in *Pseudomonas* with 125 putative LTTRs having been identified in the *Pseudomonas* genome (Schell, 1993). Several *P. aeruginosa* virulence genes have been shown to be under the control of LTTRs, for example MexT and MvfR, both of which regulate a range of virulence-associated factors, such as elastase, phospholipase, homoserine lactone and the *Pseudomonas* quinolone signal (Tian *et al.*, 2009; Wade *et al.*, 2005). The key enzyme in alginate synthesis, AlgD, has also been shown to be under the control of an LTTR, CysB (Delic-Attree *et al.*, 1997). As such, LTTRs are rapidly emerging as a key family of regulators influencing a wide range of virulence processes in *P. aeruginosa* (Reen *et al.*, 2013b).

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Abbreviations: CF, cystic fibrosis; EMSA, electromobility shift assay; LTTR, LysR-type transcriptional regulator; MCS, multiple cloning site; T3SS, type III secretion system.

Two supplementary tables and four supplementary figures are available with the online version of this paper.

In-depth functional analysis of uncharacterized host-activated LTTRs is necessary to fully elucidate the network of regulation influencing *P. aeruginosa* pathogenicity. Previously, phylogenetic analysis of LTTRs in the genus *Pseudomonas* identified several distinct clusters (Reen *et al.*, 2013a). While the analysis was based solely on amino acid sequence relationships, a pattern of interconnected regulation or cross-talk was observed. This was particularly evident in the regulation of pathogenic traits. However, for the most part these LTTRs were transcriptionally silent during co-culture experiments with host cells (Frisk *et al.*, 2004). To identify potential LTTRs that are actively involved in the host–pathogen interaction, publicly available transcriptome profiles were interrogated and *PA2877* (*PA14_26880* homologue in *P. aeruginosa* clinical isolate PA14) was shown to be upregulated in response to epithelial cells (Frisk *et al.*, 2004). To investigate its potential role in pathogenicity, PA14_26880 was characterized at both the molecular and the phenotypic level in *P. aeruginosa* PA14. This LTTR was found to have a significant influence on the expression of acute determinants, with mutation of *PA14_26880* leading to enhanced killing of the nematode acute model of infection. Our data support a role for PA14_26880 as a key regulator of virulence-associated phenotypes and pathogenesis in *P. aeruginosa*.

METHODS

Strains, plasmids and media. The strains and plasmids used in this study are listed in Table 1. All experiments were performed in wild-type *P. aeruginosa* strain UCBPP-PA14 or mutants obtained from the non-redundant PA14 insertion mutant library – PA14NR Set (Liberati *et al.*, 2006). All strains were routinely cultured in Luria–Bertani (LB; Sigma) broth at 37 °C with shaking at 150 r.p.m. unless otherwise stated. Antibiotics were added to cultures where required at the following concentrations: *Escherichia coli* – kanamycin 25 μg ml^{-1}, tetracycline 20 μg ml^{-1}, chloramphenicol 20 μg ml^{-1}; *P. aeruginosa* – tetracycline 25 μg ml^{-1}, gentamicin 20 μg ml^{-1}, streptomycin 100 μg ml^{-1}.

Table 1. Strains/plasmids used in this study

Name	Description	Reference/source
Plasmids		
pMP190	IncQ origin, low-copy-number *lacZ* fusionvector; Cm^R St^R	Spaink *et al.* (1987)
pMP190_*exsC*	Contains promoter region of *exsC* cloned into MCS	This study
pME6032	pVS1-p15A origin, $lacI^q$-Ptac expression vector, Tc^R	Heeb *et al.* (2000)
pME*bvlR*	pME6032 plasmid with *bvlR(PA14_26880)* gene cloned into MCS	This study
pMS402	Promoterless *lux* operon downstream of MCS, Tm^R	Duan & Surette (2007)
pMS-*bvlRp*	Contains promoter region of *bvlR* cloned into MCS	This study
pRK600	Mobilization plasmid helper	Keen *et al.* (1988)
pCR2.1-TOPO	PCR cloning vector, Ap^R Km^R	Invitrogen
pET28a	T7 promoter-driven His-tag protein expression vector, Km^R	Novagen
pET28abvlRCter	C-terminal His-tagged *bvlR* in pET28a	This study
Strains		
PA14 pME6032	Wild-type PA14, *P. aeruginosa* clinical isolate, containing empty plasmid pME6032	This study
PA14::*bvlR* pME6032	PA14 with Tn*5* Mariner Transposon in *bvlR*, containing empty plasmid pME6032	This study
PA14::*bvlR* pME*bvlR*	PA14 with Tn*5* Mariner Transposon in *bvlR* ORF, pME6032 plasmid with *bvlR* gene cloned into MCS	This study
PA14 pMSbvlR	PA14, containing pMS plasmid with *bvlR* promoter cloned into MCS	This study
PA14::*bvlR* pMS	PA14 with Tn*5* Mariner Transposon in *bvlR*, containing empty plasmid pMS	This study
PA14::*bvlR* pMS-*bvlRp*	PA14 with Tn*5* Mariner Transposon in *bvlR*, containing pMS plasmid with *bvlR* promoter cloned into MCS	This study
PA14 pME6032 pMP190_exsC	Previously described strain containing pMP190_exsC	This study
PA14::*bvlR* pME6032 pMP190_exsC	Previously described strain containing pMP190_exsC	This study
PA14::*bvlR* pME*bvlR* *pMP190_exsC*	Previously described strain containing pMP190_exsC	This study
PA14::*bvlA*	Wild-type PA14 with Tn*5* Mariner Transposon in *bvlA* ORF	Liberati *et al.* (2006)
E. coli HB101	Cloning and subcloning host	Sambrook & Russell (2001)
E. coli Top10	Cloning and subcloning host	Invitrogen
E. coli DH5α	Cloning and subcloning host	Boyer & Roulland-Dussoix (1969)
E. coli OP50	*Caenorhabditis elegans* non-toxic strain	Blier *et al.* (2011)
BL21-CodonPlus BL21(DE3)-RIPL	Protein expression host	Merck

Generation of plasmids used in this study. All PCRs were performed in a final volume of 20 µl using *Pfu* Proofreading polymerase (Promega) according to the manufacturer's recommendations. Primers were designed based on the PA14 genome sequence (NC_008463) and can be found in Table S1 (available in the online Supplementary Material). The PCR products were then A-tailed using 100 µM dATP (Applied Bioscience) and AccuPrime polymerase (Roche Applied Science) and incubated at 94 °C for 2 min followed by 68 °C for 20 min, after which the PCR products were cloned into pCR2.1-TOPO (Invitrogen) according to the manufacturer's recommendations. All constructs were verified by sequence analysis (GATC Biotech), and subsequently cloned into their respective host plasmids and finally transferred from *E. coli* DH5α (Boyer & Roulland-Dussoix, 1969) to *P. aeruginosa* by triparental mating using the helper plasmid pRK600 as described by de Lorenzo & Timmis (1994) and Keen *et al.* (1988).The T4 DNA ligase and all restriction enzymes used in this study were purchased from Roche Applied Sciences and were used according to the manufacturer's recommendations.

To generate plasmid pME*bvlR*, the *PA14_26880* ORF was amplified using primers bvlR OEF and bvlR OER, which contain incorporated *Bgl*II and *Kpn*I restriction sites, respectively, and was ligated into the pME6032 multiple cloning site (MCS) downstream of the Ptac promoter (Heeb *et al.*, 2000). The vector was then transformed by heat shock into *E. coli* Top10 Cloning and subcloning host (Invitrogen).

To generate an *exsC* promoter expression construct, a 340 bp fragment upstream of *exsC* was amplified with exsC TFF and exsC TFR, which incorporated the *Xba*I and *Kpn*I restriction sites, respectively. The *Xba*I–*KpnI exsC* fragment was cloned into *Xba*I–*KpnI*-digested pMP190 (Spaink *et al.*, 1987) to produce pMP190_*exsC*.

To construct a plasmid expressing a His-tagged BvlR protein, the *bvlR* ORF was amplified using primers BvlR His F and BvlR His R, which incorporate *Nco*I and *Hin*dIII restriction sites, respectively. Finally, the amplicon was cloned into the MCS of pET28a to yield pET28abvlRH6C, which expressed a C-terminal His-tagged BvlR protein.

Transcriptional fusions to *luxCDABE* were generated by cloning the promoter regions of *PA14_26880*, amplified by PCR using primer sets bvlR TFF and BvlR TFR. The amplicon was flanked by *Xho*I–*Bam*HI restriction sites, allowing it to be ligated into the sites *Xho*I–*Bam*HI of pMS402 (Duan & Surette, 2007). Luminescence was measured over time using a Tecan GENios Microplate Reader.

***β*-Galactosidase assay.** For the β-galactosidase assay, *P. aeruginosa* cultures were grown overnight in LB broth supplemented with appropriate antibiotics. IPTG was added at a concentration of 1 mM for all pME6032 derivative plasmids. Cells were diluted to an OD_{600} of 0.05 in 20 ml and incubated at 37 °C at 150 r.p.m. Cells were recovered at the exponential phase of growth and β-galactosidase assays were performed as described by Miller (1972). Data are the mean of three independent biological replicates.

Microcolony formation assay. Artificial sputum medium (ASM) was prepared as described by Sriramulu *et al.* (2005). ASM (1 ml) was inoculated to an OD_{600} of 0.05 with an overnight culture of *P. aeruginosa* PA14 in 24-well cell-culture plates (Sarstedt). IPTG was added at a concentration of 1 mM for all pME6032 derivative plasmids. Plates were then incubated at 37 °C at 150 r.p.m. for 72 h. Three independent biological replicates were performed, and representative results are shown.

***Caenorhabditis elegans* assays.** The *C. elegans* assay was performed as described by Blier *et al.* (2011). In brief, *C. elegans* was maintained under standard culturing conditions at 22 °C on nematode growth medium (all per litre, 3 g NaCl, 2.5 g peptone, 17 g agar, 5 mg cholesterol, 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$, 25 ml 1 M KH_2PO_4) agar plates with *E. coli* OP50 as a food source (Sulston & Hodgkin, 1988). Synchronous cultures of worms were generated. Pathogen lawns used for *C. elegans* survival assays were prepared by spreading 50 µl *P. aeruginosa* strains corrected to an OD_{600} of 1 (mutant vs. complemented). The plates were incubated overnight at 37 °C and then placed at room temperature for 4 h. Between 15 and 20 L4 synchronized worms were harvested with M9 solution, placed on 35 mm assay Petri dishes and incubated at 22 °C. Worm survival was scored at 1 h, 24 h and each subsequent day, using an Axiovert S100 optical microscope (Zeiss) equipped with a Nikon digital camera DXM 1200F (Nikon Instruments). The worms were considered dead when they remained static without grinder movements for 20 s. Standard ethical procedures were used. The results are expressed as the percentage of living worms and are the mean of three independent biological assays.

Attachment assay. Due to the viscous nature of ASM, it was not feasible to accurately determine levels of bacterial attachment in this medium. Therefore, M63 medium was prepared as described by O'Toole & Kolter (1998) and used to assess bacterial attachment. Strains were inoculated from a fresh overnight culture into 1 ml of M63 broth in 24-well microtitre plates (Starstedt) at an OD_{600} of 0.25. IPTG was added to the medium at a concentration of 1 mM. Cultures were incubated at 37 °C for 1 h statically. OD_{600} was measured to ensure no difference in growth. Cultures were removed and wells washed three times with 1.2 ml LB. Then, 1.3 ml of 0.1 % crystal violet (Sigma) was added and incubated at room temperature for 10 min. The crystal violet was removed and the wells were washed five times with distilled H_2O. Plates were then dried at 37 °C for 2 h. Then, 1.5 ml of 96 % EtOH was added to each well and incubated at room temperature for 15 min. One hundred microlitres was then taken from each well and added to a clean 96-well plate (Corning) and OD_{570} was measured.

Flow cell biofilm assay. Flow cell chambers with individual channel dimensions of $1 \times 4 \times 40$ mm were used. The flow system was assembled and prepared as described by Gjermansen *et al.* (2005). Overnight cultures of *Pseudomonas* strains were inoculated as single colonies into 5 ml of M63 medium (O'Toole & Kolter (1998) and shaken (180 r.p.m.) overnight at 37 °C. Bacteria were grown in M63 medium throughout the flow cell experiment. The flow chambers were inoculated by injecting 250 µl overnight culture diluted to an OD_{600} of 1 into each flow channel using a small syringe. After inoculation, the flow channels were left without flow for 1 h, after which medium flow was started using a Watson Marlow 205S peristaltic pump. The mean flow velocity in the flow chambers was 0.2 mm s^{-1}, corresponding to a laminar flow with a Reynolds number of 0.02. The flow cell biofilm system was incubated at 30 °C throughout the experiment. Bacteria were stained using 5 µM SYTO 9 (Invitrogen). Visualization of the biofilm was performed using a Zeiss LSM5 confocal laser scanning microscope equipped with the 488 nm laser lines to excite SYTO 9. Three independent biological replicates were performed. Images were processed using the Imaris 6.0.2 Software suite.

RNA isolation, global expression analysis, cDNA synthesis and quantitative real-time PCR. RNA was extracted from exponential-phase cultures (OD_{600} of 0.7–0.8) of PA14 pME6032, PA14::*bvlR* pME6032 and PA14::*bvlR* pME6032_bvlR using the RNeasy minikit (Qiagen). Genomic DNA was removed using TURBO DNase (Ambion). Isolated RNA was sent to DNAVision for further analysis. RNA quality was assessed using a Bioanalyser Agilent 2100 at DNAVision. cDNA synthesis, fragmentation and terminal labelling preceded hybridization on Affymetrix GeneChip *P. aeruginosa* genome arrays according to the Affymetrix guidelines. GeneSpring GX software was used to analyse raw data following the manufacturer's guidelines.

In brief, the raw data were normalized using the robust multiarray average algorithm. An empirical Bayesian unpaired comparison (moderated *t*-test, $P<0.05$) was carried out to generate a list of genes with significantly altered expression between the test strains of greater than twofold.

For quantitative real-time PCR analysis, cDNA was synthesized using AMV reverse transcriptase (Promega) and random primers (Invitrogen). Real-time primers were designed utilizing the Universal Probe Library Assay Design Center (UPL, Roche). Real-time PCR was conducted on a Chromo4 Continuous Fluorescence Detector (MJ Research) using FastStart TaqMAN Probe Master and probes from the Universal ProbeLibrary (UPL, Roche) according to the manufacturer's recommendations. All data presented are relative to the housekeeping gene *proC*, and are the mean of three independent biological replicates.

His-tag purification of BvlR. The pET28abvlRH6C construct was transformed into the *E. coli* expression host strain BL21-CodonPlus BL21(DE3)-RIPL (Merck) and grown at 37 °C with shaking at 150 r.p.m. in 250 ml LB medium containing kanamycin (50 mg ml^{-1}) until an OD_{600} of 0.8 was reached. At this point 1 mM IPTG was added to the culture to induce expression of the His-tagged BvlR protein. After 4 h, cells were harvested by centrifugation at 5000 ***g*** at 4 °C and stored at −70 °C overnight. Cell pellets were subsequently thawed and resuspended in CelLytic B II buffer (10 ml per gram of cell paste) with 50 units benzonase ml^{-1}, 0.2 mg lysozyme ml^{-1} and 100 µl of Protease Inhibitor Cocktail (all concentrations relative to 1 g of cell paste). Protein extract was applied to a Poly-4 Prep Chromatography Column (Bio-Rad) containing 1 ml of HIS-Select Nickel Affinity Gel. The gel was washed with 2 ml of sterile de-ionized water and equilibrated with 5 ml of wash buffer (100 mM HEPES, pH 7.5, 10 mM imidazole). Following washing and equilibration steps, the crude protein extract (10 ml) was passed through the column by gravity flow. Proteins which bound to the resin were washed twice with 5 ml wash buffer and purified His-tagged BvlR protein was eluted in wash buffer containing imidazole at the following concentrations (1, 10, 50, 100, 250, 500 and 750 mM). The purity of the purified BvlR protein was assessed by SDS PAGE as described by Sambrook & Russell (2001). Protein concentrations were determined by the Bio-Rad protein assay (Bradford, 1976) and purified protein aliquots were used for electromobility shift assays (EMSAs). All chemicals were purchased from Sigma Aldrich unless stated otherwise.

EMSA. First, the labelled promoter DNA fragments were generated by amplification of the promoter fragments with infrared-labelled primers (Eurofins MWG Operon) (Table 1) using *Pfu* Proofreading polymerase (Promega), according to the manufacturer's recommendations in a total volume of 20 µl. Amplified DNA fragments were visualized by agarose gel electrophoresis and subsequently purified using a gel extraction kit (Qiagen), according to the manufacturer's recommendations. The *bvlR/bvlA* IRF and *bvlR/bvlA* IRR primer set was used to construct the *blvA–bvlR* intergenic region, whereas the truncated *blvA–bvlR* intergenic region was amplified using *bvlR/bvlA* IT IRF and *bvlR/bvlA* IT IRR primers. The *oprF–sigX* intergenic region was amplified using the primers OprF IRF and OprF IRR. The primer sets use to amplify other promoter regions included in this study are presented in Table S1 The final EMSA reaction volume was 20 µl, containing varying concentrations of purified BvlR protein (50–2000 nM) in the presence of 10 fmol of labelled promoter DNA fragment in EMSA binding buffer [20 mM HEPES, pH 7.6, containing 30 mM KCl, 5 mM $(NH_4)_2SO_4$, 1 mM EDTA, 1 mM DTT, 0.2 % (w/v) Tween 20 and 5 µg poly[d(I-C)] ml^{-1}]. The reaction was incubated at 28 °C for 40 min, and samples were then separated by electrophoresis on 6 % (v/v) native polyacrylamide gel and visualized on an Odyssey Infrared Imaging System (Li-COR Biosciences). Control binding reactions were performed with extracts from *E. coli* BL21-CodonPlus BL21(DE3)-RIPL cells harbouring the pET28a vector control.

Bioinformatic analysis. DNA sequence was obtained from the PA14 genome sequence (NC_008463) and used as bait in sequential BLASTN and BLASTP searches. Potential BvlR binding motifs were identified using the DNA Motif Search tool at www.pseudomonas.com. All amino acid sequences were aligned with CLUSTAL W and phylogenetic analysis was performed using the MEGA5 software program. Trees were generated using the neighbour-joining algorithm (p-Distance) using as outgroup *P. aeruginosa* PA2206, a non-homologous LTTR. Minimum bootstrap support values $\geqslant 95$ % were enforced for this analysis.

Statistical analysis. For the *C. elegans* assay, survival was calculated by using the Kaplan–Meier method, and survival differences were tested for significance by using the log-rank test (GraphPad Prism version 4.0, GraphPad Software) over three independent biological replicates. For all other experiments outlined here, statistical analysis was performed using a two-tailed paired Student's *t*-test. Differences were considered significant if the *P*-value was $\leqslant 0.05$ over three independent biological replicates.

RESULTS

A new repressor (PA14_26880) of the type III secretion system

The process of infection by *P. aeruginosa* is primarily characterized by two distinct phases. Initial colonization through expression of acute virulence determinants is followed by a switch to a chronic biofilm state that is practically impossible to eradicate. The involvement of LTTRs in this process remains poorly understood in spite of the fact that they represent the largest family of transcriptional regulators in *P. aeruginosa*. Analysis of the publicly available transcriptome datasets revealed that *PA2877* (*PA14_26880* homologue in *P. aeruginosa* clinical isolate PA14) is transcriptionally altered in response to epithelial cell contact, suggesting that it may play a role in *P. aeruginosa* pathogenicity. Transcriptional analysis was used to determine if PA14_26880 exerted a regulatory influence over the acute-associated type III secretion system (T3SS). The promoter region of the *exsCDBA* operon, containing the T3SS master regulator ExsA, was cloned upstream of a promoterless *lacZ* gene into pMP190, resulting in pMP190_*exsC*. β-Galactosidase assays verified that T3S was elevated 1.7-fold (Student's *t*-test, $P<0.05$) in the PA14_26880 transposon mutant derived from the non-redundant PA14 insertion mutant library (Liberati *et al.*, 2006) (Fig. 1a). This elevated transcription was restored to wild-type levels through complementation with the PA14_26880 ORF cloned into the inducible plasmid pME6032 (Fig. 1a).

PA14_26880 promotes tight microcolony formation

Having established a role for *PA14_26880* in the regulation of T3S, the possibility that this transcriptional regulator

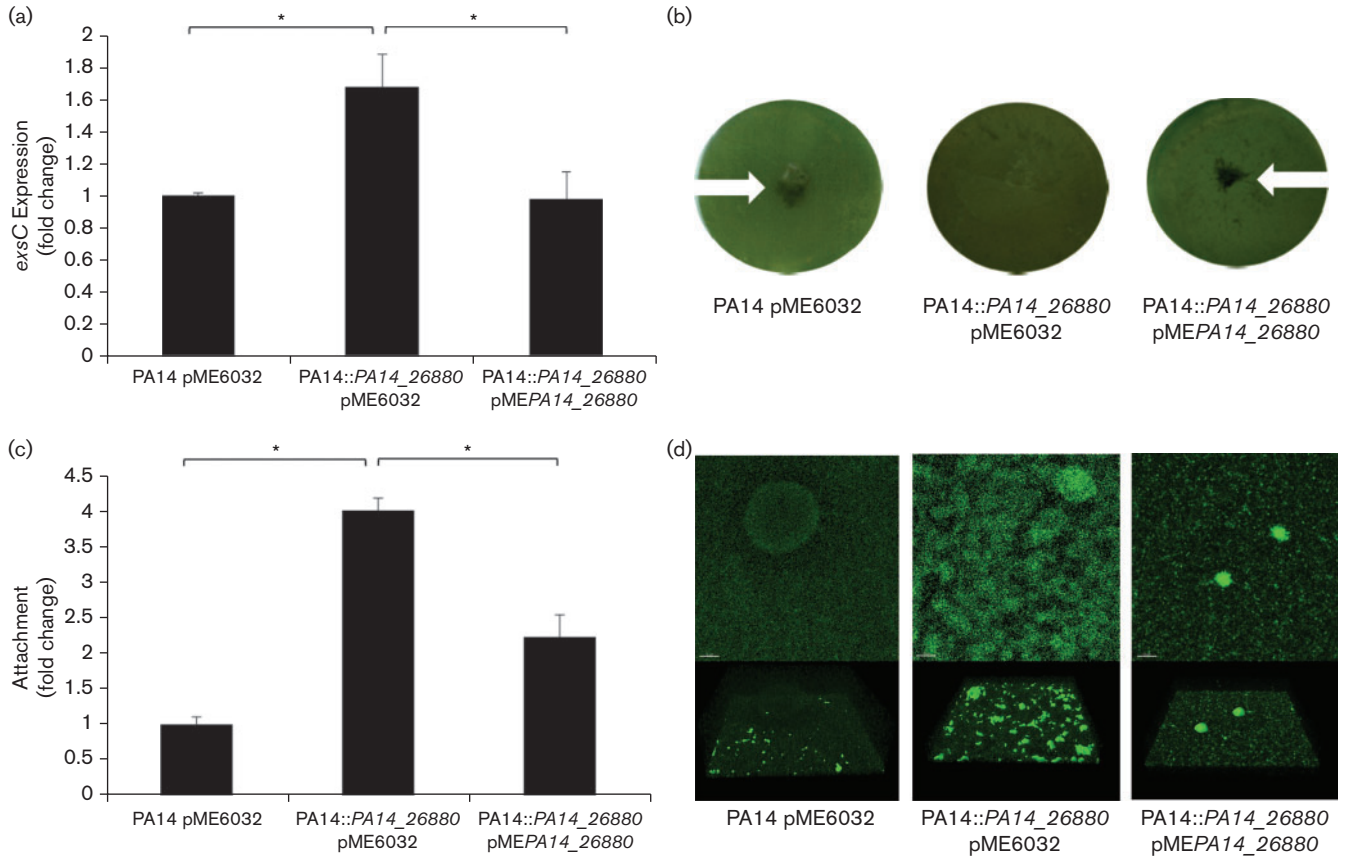

Fig. 1. (a) Elevated levels of T3S in a PA14_26880 mutant. Expression from pMP190_*exsC* in PA14 pME6032, PA14 : : *PA14_26880* pME6032 and PA14 : : *PA14_26880* pME *PA14_26880* was assessed by performing β-galactosidase assays on cells that were recovered at the exponential phase of growth. Fold change is relative to wild-type levels. All measurements are in Miller units. Data shown are mean values of three biological experiments with SD as error bars. *Student's *t*-test, $P < 0.05$. (b) PA14_26880 has a role in tight microcolony formation. Tight microcolony formation was assessed by growing strains in ASM for 72 h in 24-well plates. Tight microcolony formation is seen as dark non-surface-attached aggregates, which are highlighted by a white arrow. These tight microcolonies project up into the well but are not attached to any surface. Complete loss of tight microcolony formation is observed in PA14 : : *PA14_26880* pME6032. The image shown is a representative of at least three independent biological replicates. (c) PA14_26880 plays a role in attachment. Levels of bacterial attachment were assessed using a crystal violet staining assay. Increased levels of attachment observed in PA14 : : *PA14_26880* pME6032. *Student's *t*-test, $P < 0.05$. (d) PA14_26880 plays a role in attachment. To confirm the hyperattachment phenotype observed in the crystal violet assay and to ensure that this was not due to increased production of components of the biofilm matrix, flow cell biofilm analysis was performed. This analysis confirmed that a PA14_26880 mutant indeed attaches to an abiotic surface at a much higher level than wild-type cells. Images were taken after 24 h of incubation. Cells were stained with the DNA-specific SYTO9 dye. Images were processed using the Imaris 6.0.2 Software suite. Bars, 15 μm.

might also modulate biofilm formation was subsequently investigated. Biofilm formation and T3S have previously been shown to be inversely regulated, although the mechanism underpinning this observation has not been fully elucidated (Furukawa *et al.*, 2006). *P. aeruginosa* has been shown to form a tight microcolony in the CF lung. This behaviour differs from the classical definition of a biofilm as surface attachment is not required (Sriramulu *et al.*, 2005; Worlitzsch *et al.*, 2002). The influence of PA14_26880 on this host-specific mode of biofilm formation was assessed in a synthetic medium that mimics the sputum found in the CF lung (Sriramulu *et al.*, 2005). A PA14_26880 transposon mutant was unable to form tight microcolonies in this ASM. Complementation with pME*bvlR* restored the ability of a PA14_26880 mutant to form tight microcolonies. These data suggest that PA14_26880 is integral to the formation of non-surface-attached biofilms such as those found in the CF lung (Fig. 1b). Interestingly, the *PA14_26880* mutant exhibited increased levels of abiotic surface attachment, which was restored to wild-type levels through complementation (Fig. 1c). Flow cell biofilm analysis confirmed the hyperattachment phenotype associated with a *PA14_26880* mutant (Fig. 1d). Assays of extracellular matrix components such as rhamnolipid and extracellular DNA (data not

shown) were performed and demonstrated no difference between the wild-type and mutant, suggesting that this increased attachment is not due to an alteration in the production of biofilm matrix components. This increased attachment phenotype is consistent with the *PA14_26880* mutant cells being unable to self-aggregate in a tight microcolony. The negative regulation of T3SS and promotion of tight microcolony formation by PA14_26880 suggests a role in the chronic phase of *P. aeruginosa* infection within the host. Given the role of PA14_26880 in these key virulence phenotypes it is hereafter referred to as BvlR (Biofilm and Virulence LysR Regulator).

BvlR plays a key role in virulence in the *C. elegans* model of infection

Having established that BvlR plays a role in the modulation of key virulence determinants, it was hypothesized that it may play a role in the overall pathogenic potential of *P. aeruginosa*. To investigate this, the nematode model of infection *C. elegans* was utilized. To ensure isogenicity, the *bvlR* mutant was compared with the complemented pME*bvlR* strain. The presence of BvlR led to a significant reduction in virulence at each time point (Student's *t*-test, $P<0.01$) (Fig. 2), suggesting a significant role in *P. aeruginosa* pathogenesis. Therefore, BvlR was functionally characterized to elucidate the molecular mechanisms underlying this key role in pathogenicity.

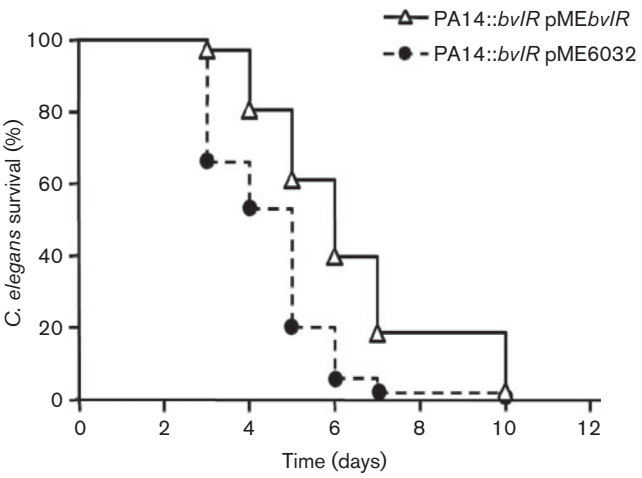

Fig. 2. BvlR modulates virulence in the *Caenorhabditis elegans* model of infection. Percentage *C. elegans* survival is shown on the *y*-axis and length of survival is shown on the *x*-axis. The mean results of three independent biological experiments are shown. Between 45 and 60 L4 synchronized worms were used per strain for each biological replicate. The observed difference for strain PA14 : : *bvlR* pME*bvlR* is significant (Student's *t*-test, $P<0.01$) for each time point of assay.

BvlR negatively regulates the expression of a divergent gene *bvlA* and auto-regulates its own expression

To identify the pathway(s) through which BvlR mediates expression of virulence determinants in *P. aeruginosa*, it was first decided to assess if BvlR fulfilled the expected molecular characteristics associated with a 'classical' LTTR. LTTRs are typically capable of negative auto-regulation and positively influence the transcription of divergently transcribed genes in close genomic proximity. These divergently transcribed targets are often responsible for the LTTRs, effect on virulence (Maddocks & Oyston, 2008; Schell, 1993). To investigate whether BvlR was capable of auto-regulation, the promoter region of *bvlR* was cloned into a plasmid, pMS402, which contains a promoterless *−lux* fusion. In a *bvlR* mutant expression of *bvlR* was significantly higher (Student's *t*-test, $P<0.05$) than that observed in the wild-type over an 8 h time period (Fig. 3a), indicating that *bvlR* was capable of auto-regulation.

Divergently upstream from *bvlR* is *PA14_26870* (*bvlA*), a gene assigned a hypothetical function. Bioinformatic analysis showed that *bvlR* and *bvlA* gene synteny is conserved in other *Pseudomonas* species. Moreover, a high degree of conservation is observed in the intergenic region between *bvlR* and *bvlA*, indicating that regulatory elements of significance may be present within this region. Quantitative real-time PCR was used to demonstrate that BvlR exerted a strong negative regulatory influence over *bvlA* (Student's *t*-test, $P<0.05$) (Fig. 3b). The negative influence of BvlR on the expression of *bvlA* is contrary to the observed regulatory features typically assigned to an LTTR. This evidence suggests that BvlR may be an LTTR that functions predominantly as a transcriptional repressor, few of which have previously been described (Maddocks & Oyston, 2008).

BvlR exerts its regulatory influence over *bvlA* through direct binding to the *bvlR–bvlA* intergenic region

Typically LTTRs exhibit their regulatory influence over a divergently transcribed gene through direct binding (Maddocks & Oyston, 2008). A His-tagged BvlR expression construct was generated (pET28a*bvlR*H6C) and the BvlR regulator was subsequently purified as a monomer of 34.5 kDa by nickel affinity column chromatography (Fig. S1). An EMSA was performed to assess if the transcriptional influence that BvlR was exerting over *bvlA* was due to direct binding. Purified His-tagged BvlR fusion protein caused a mobility shift in the presence of an infrared-labelled DNA probe spanning the *bvlR–bvlA* intergenic region (Fig. 4b). These data demonstrate that BvlR is binding to the *bvlR–bvlA* intergenic region. As LTTRs typically bind a specific motif, T-N_{11}-A, the *bvlR–bvlA* intergenic region was analysed for such a motif. A single T-N_{11}-A (TCCCGAATAGAGA) motif was identified in the *bvlR–bvlA* intergenic region (Fig. 4a). To investigate if this motif was essential for direct binding, EMSA was performed using a truncated *bvlR–bvlA* intergenic region, amplified using the

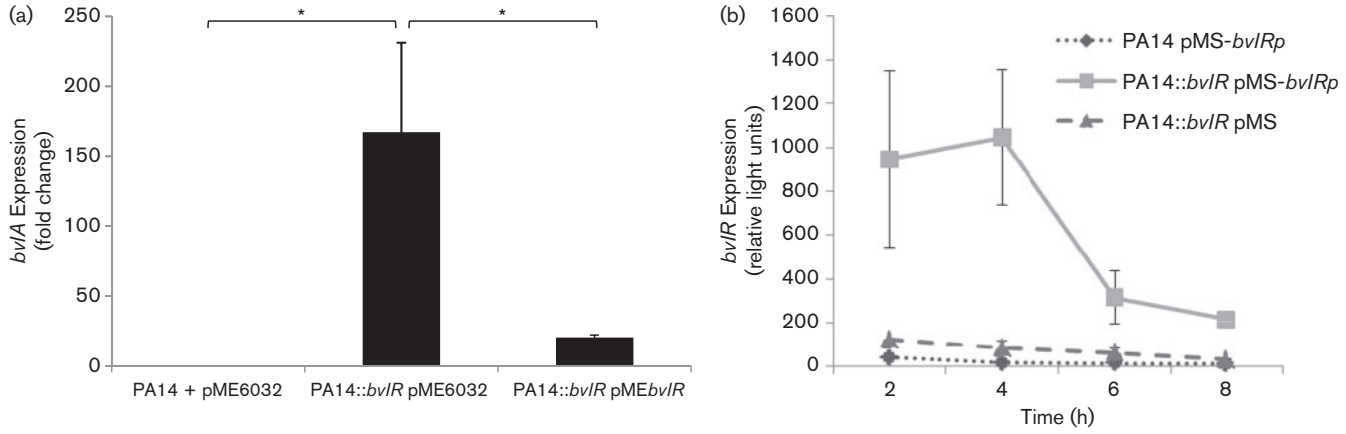

Fig. 3. (a) BvlR regulates *bvlA* expression levels. Quantitative real-time PCR was performed using primers specific to *bvlA*. Fold change in presented relative to the housekeeping gene *proC*. *Student's *t*-test, $P < 0.05$. (b) BvlR demonstrates negative auto-regulation. Expression of *bvlR* over time measured in relative light units using a modified pMS402 that contains a *bvlR* promoter–*lux* fusion conjugated to PA14 pME6032 and PA14 : : *bvlR* pME6032. All light readings were made relative to optical density. The data are the mean of three independent biological replicates.

primer set *bvlR/bvlA* IT IRF and *bvlR/bvlA* IT IRR, which did not contain this potential binding site. The purified His-tagged BvlR fusion protein did not cause a mobility shift in the presence of this amplicon encompassing the sequence from −31 to −249 bp downstream of the *bvlA* transcriptional start site (Fig. 4c), demonstrating that BvlR binding requires the TCCCGAATAGAGA motif. As BvlR and BvlA are divergently transcribed the *bvlR–bvlA* intergenic region encompasses the promoter regions of both genes. Thus, the directing binding to this intergenic region is likely to be not only responsible for the effect of BvlR on *bvlA* transcription but also for the auto-regulation of *bvlR* expression. As *bvlA*

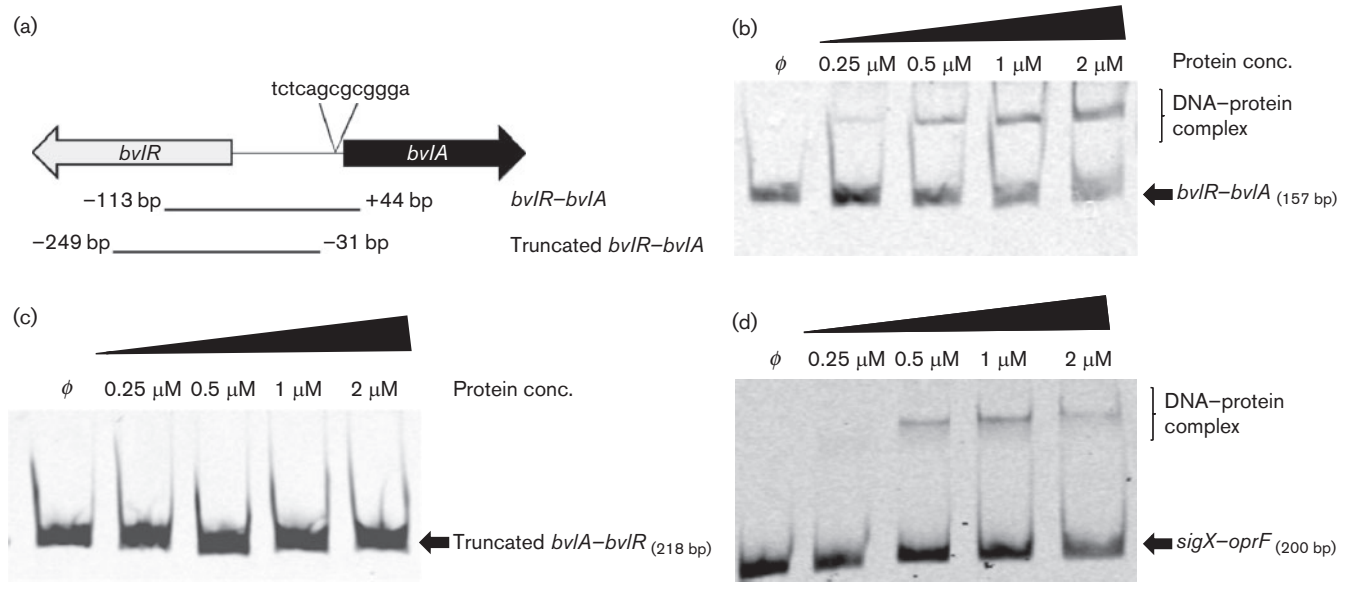

Fig. 4. Direct binding of BvlR to the bvlA–bvlR intergenic region. (a) The genomic location of both infrared-labelled fragments with respect to *bvlR* and *bvlA*. (b) His-tag-purified BvlR causes a mobility shift in the presence of the *bvlA* upstream region. (c) No shift was observed with a truncated *bvlA* upstream region which lacks the BvlR binding site. (d) Direct binding of BvlR to the *sigX–oprF* region. His-tag purified BvlR causes a mobility shift in the presence of the *sigX–oprF* intergenic region. The concentration of His-tagged BvlR used in each assay is indicated above the gel image. Infrared-labelled DNA at 10 fmol was used.

is a direct transcriptional target of BvlR it could be responsible for the phenotypes associated with a *bvlR* mutant. However, a *bvlA* mutant failed to influence T3S, attachment or tight microcolony formation, indicating that *bvlA* is not responsible for the BvlR-associated phenotypes identified in this study (data not shown). That BvlA does not appear to play a role in any of the identified BvlR phenotypes suggests that other targets of BvlR may be responsible for the observed phenotypes, consistent with the emerging role of LTTRs as global regulators of gene expression.

BvlR regulation controls virulence-associated gene expression

To identify other transcriptional targets of BvlR that could explain the observed phenotypes, global transcriptomic analysis was performed. To ensure isogenicity, the *bvlR* mutant was compared with the complemented strain for transcriptomic analysis. Approximately 5.6 % of the genes (307 genes of the 5500 *P. aeruginosa* PAO1 probe sets on the microarray) demonstrated significantly altered expression greater than twofold (moderated *t*-test, $P<0.05$) (Table S2). The changes in gene expression ranged from −10.6- to −2-fold. BvlR negatively influenced the expression of all of the genes in the transcriptome dataset, further emphasizing BvlR's potential role as a transcriptional repressor. A number of genes associated with virulence were repressed by BvlR (Table 2), confirming the influence of BvlR on the pathogenicity of *P. aeruginosa*. Genes involved in metabolite transportation were also highly represented within the transcriptome dataset. To establish further the role of BvlR, the transcriptome profile was investigated for links between the changes in gene expression and *bvlR*-associated virulence phenotypes.

BvlR modulates expression genes associated with surface attachment. Expression of key genes of the *cupA* operon was induced in the *bvlR* mutant. The *cupA* operon encodes components and assembly factors of a fimbrial structure that are known to play a key role in surface attachment. It has been shown that increased expression of this operon can lead to a hyperattachment phenotype (Kulasekara *et al.*, 2005; Meissner *et al.*, 2007; Vallet *et al.*, 2001). This corresponds with the increased attachment observed in a *bvlR* mutant. Quantitative real-time PCR analysis of *cupA2* and *cupA3* confirmed the initial finding of the transcriptome dataset that the *cupA* operon was indeed upregulated in a *bvlR* mutant (Fig. 5a). It is thought that expression of the *cup* operon is specific to particular niches such as attachment to specific biological surfaces (Kuehn *et al.*, 1992). A number of genes associated with alginate biosynthesis were also modulated in the transcriptome. This finding was confirmed by quantitative real-time PCR using primers specific to *algK* (Fig. 5b). It is known that alginate is capable of modulating biofilm architecture, although it is not essential for biofilm formation (Lizewski *et al.*, 2004). Attachment to a substratum has been shown to induce expression of genes in the alginate biosynthetic pathway (Boyd & Chakrabarty, 1994). Based on the influence of surface attachment on alginate expression, the increased expression of key genes in

Table 2. Virulence genes negatively regulated greater than twofold by BvlR identified through microarray analysis (moderated *t*-test, $P<0.05$)

ORF ID	Gene name	Description	Fold change
Genes associated with attachment and biofilm			
PA3541	*alg8*	Alginate biosynthesis protein	2.5
PA3548	*algI*	Alginate *O*-acetyltransferase	2.1
PA3549	*algJ*	Alginate *O*-acetyltransferase	2.0
PA3543	*algK*	Alginate biosynthetic protein	10.6
PA3547	*algL*	Poly(β-D-mannuronate) lyase precursor	2.1
PA2129	*cupA2*	Chaperone	2.2
PA2132	*cupA5*	Chaperone	2.4
PA4084	*cupB3*	Usher	7.2
PA4083	*cupB4*	Chaperone	2.6
PA4082	*cupB5*	Adhesive protein	4.1
PA4081	*cupB6*	Fimbrial subunit	3.2
PA2235	*pslE*	Exopolysaccharide biosynthesis	2.3
PA2236	*pslF*	Exopolysaccharide biosynthesis	2.4
Genes associated with virulence			
PA4813	*lipC*	Lipase	8.4
PA3319	*plcN*	Non-haemolytic phospholipase C precursor	2.6
PAO843	*plcR*	Phospholipase accessory protein	2.0
PA1148	*toxA*	Exotoxin A precursor	2.4
PAO707	*toxR*	Transcriptional regulator	2.4

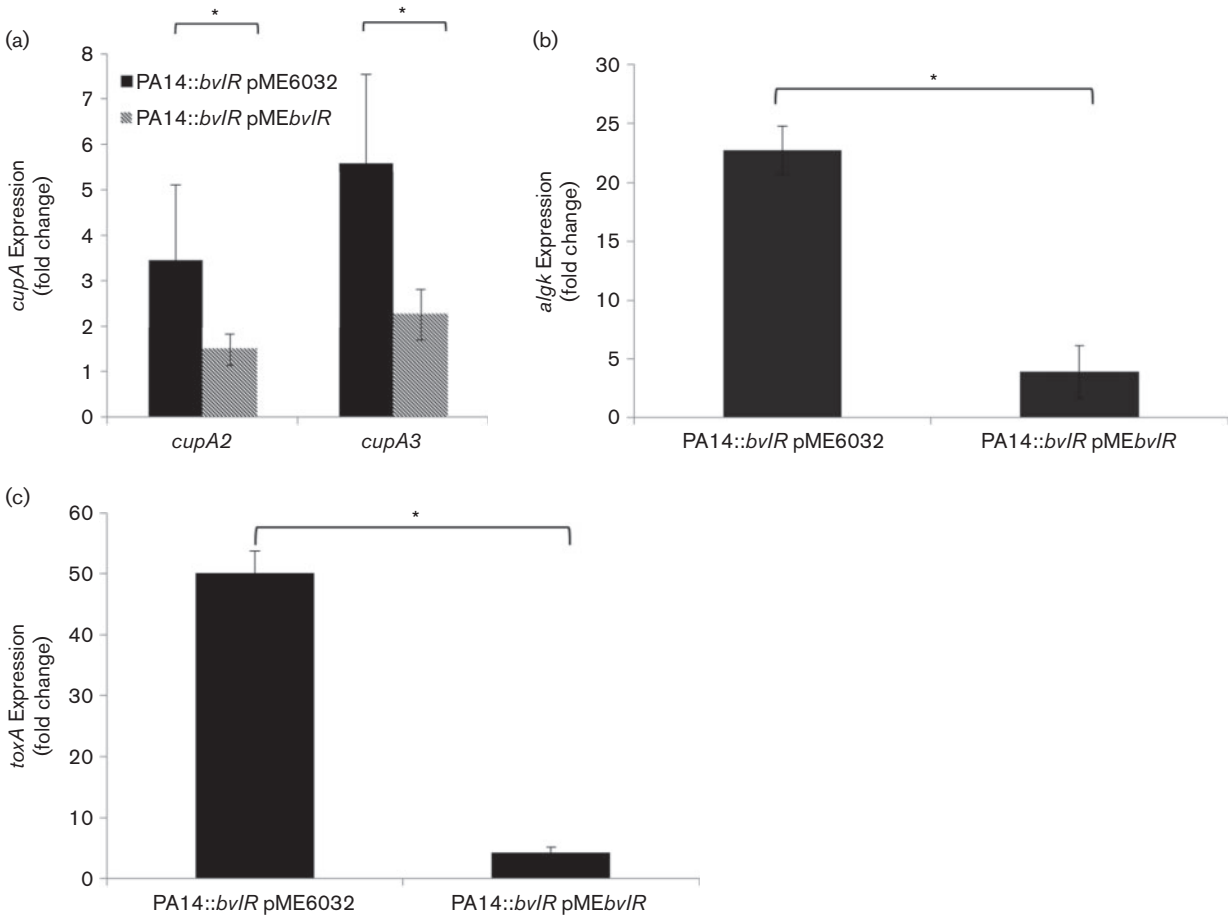

Fig. 5. BvlR is a repressor of virulence-associated gene expression. (a) BvlR negatively regulates *cupA* gene expression. Expression of key genes in the *cupA* operon was assessed using quantitative real-time PCR. Primers specific to *cupA2* and *cupA3* demonstrated a significant repression of operon expression when *bvlR* is expressed (Student's *t*-test, $P<0.05$). (b) BvlR modulates *algK* transcription. Quantitative real-time PCR was used to determine that expression of *algK* is reduced sixfold when an active copy of *bvlR* is expressed (Student's *t*-test, $P<0.05$). (c) BvlR negatively regulates *toxA* expression. Quantitative real-time PCR analysis of exotoxin A production demonstrates that expression of *bvlR* leads to a 14-fold reduction in *toxA* transcript levels (Student's *t*-test, $P<0.05$). RNA was extracted from exponential-phase cultures. All values are presented relative to the housekeeping gene *proC*. Data shown are mean values of three biological experiments with SD as error bars. *Student's *t*-test, $P < 0.05$.

the alginate biosynthetic operon in the *bvlR* mutant may be a result of the increased surface attachment associated with the *bvlR* mutant.

BvlR negatively regulates exotoxin A expression. BvlR expression also modulated *toxA* expression in the transcriptome dataset. Exotoxin A (*toxA*) represents one of the key virulence factors through which *P. aeruginosa* is capable of killing *C. elegans* (Fogle *et al.*, 2002; Matsumoto *et al.*, 1999). ToxA is an ADP-ribosyltransferase that can inhibit protein synthesis (Iglewski & Kabat, 1975). Various animal models have demonstrated the importance of exotoxin A in *P. aeruginosa* infection, with *toxA* mutants typically being less virulent than their wild-type counterparts (Fogle *et al.*, 2002; Matsumoto *et al.*, 1999). To validate the transcriptome results, expression levels of exotoxin A were determined using quantitative real-time PCR analysis. A 14-fold reduction in *toxA* levels was observed when BvlR is present (Student's *t*-test, $P<0.05$) (Fig. 5c). The observed effect of BvlR on pathogenicity in the *C. elegans* model is probably mediated through the reduction in exotoxin A levels. The positive regulator of exotoxin A, ToxR, was also found to be downregulated twofold in the transcriptome dataset, suggesting that BvlR regulatory influence over *toxA* may occur through cross-talk between these distinct transcriptional regulators.

The transcriptome dataset highlights a network of genes that are transcriptionally repressed by BvlR. Although this further supports the evidence that *bvlR* encodes an LTTR repressor that is capable of downregulating genes associated with acute virulence, while promoting the formation of the self-aggregating tight microcolonies of a mucoid CF

lung, the molecular interactions underpinning this regulation need to be established.

BvlR role in virulence may be both direct and indirect

To determine the pathway through which BvlR exerts its effects on gene expression linked to virulence, candidate promoters were examined for binding affinity with purified BvlR protein. A dual approach was taken to the selection of these promoters, focusing on (a) virulence-associated genes from the transcriptome profiling and on (b) those with highly similar T-N_{11}-A motifs to the BvlR box. Bioinformatic analysis of the *P. aeruginosa* genome sequence failed to identify complete BvlR binding motifs, other than that in the *bvlR–bvlA* intergenic region. By systematically reducing the stringency to allow for mismatches, a bank of potential BvlR binding candidates was generated, which included intergenic regions upstream of virulence-associated genes such as *exsC*, *oprF* and *algQ* (Fig. S2). In addition, several promoters in which motifs closely related to the BvlR motif were identified were also included in this analysis, although these loci were not present in the transcriptome dataset.

Infrared primers were designed to each of the regions that contained a partial BvlR binding motif. These regions were then amplified and EMSA binding analysis was performed (Fig. S3). Purified BvlR was seen to cause a mobility shift for one of the potential binding targets, the *sigX–oprF* intergenic region (Fig. 4d). The BvlR binding motif in this *sigX–oprF* intergenic region had among the highest sequence similarity of all the potential candidates, with 11 of the 13 bases being conserved with the BvlR binding motif in the *bvlR–bvlA* intergenic region. While this might suggest that the interaction of purified BvlR protein with DNA is highly specific in the absence of a recognized ligand, it must be noted that other promoters with the same degree of conservation did not bind BvlR (Fig. S3). Perhaps surprisingly, although consistent with the transcriptome profiling, no significant difference in *oprF* transcription levels was observed between PA14 pME6032, PA14 : : *bvlR* pME6032 and PA14 : : *bvlR* pME*bvlR* in LB broth (data not shown).

Apart from the *sigX–oprF* promoter region, BvlR was not found to interact with the other promoters tested in this study, even though several contained highly similar motifs to that in the *bvlR–bvlA* intergenic region to which BvlR has been shown to bind. These include the promoters of several virulence-associated genes which were altered in the transcriptome profile, suggesting that at least some of the effects attributed to BvlR may be the result of indirect regulation. Notwithstanding this, it is well known that the DNA binding specificity and transcriptional influence of many LTTRs are significantly altered by the presence of a specific ligand (Maddocks & Oyston, 2008). The strong phenotypic profile associated with a BvlR mutant suggests that BvlR's direct binding targets are indeed likely to extend beyond those confirmed *in vitro*. Future work will focus on the identification of a specific ligand which may be central to the interaction of BvlR with direct transcriptional targets.

DISCUSSION

The data presented demonstrate that the LTTR BvlR is a novel virulence regulator that plays a key modulatory role in the expression of a wide range of virulence determinants. BvlR is one of the few LTTRs whose regulation is altered in response to eukaryotic cells (Frisk *et al.*, 2004), indicating a potential role for BvlR in pathogenicity. *P. aeruginosa* infection is characterized by two distinct phases, the acute state and chronic state. Acute infection involves the production of a wide range of virulence determinants and a motile mode of growth. BvlR expression was shown to repress the T3SS, one of the main exotoxin secretion systems associated with acute infection (Jin *et al.*, 2011; Mulcahy *et al.*, 2006; Yahr *et al.*, 1995; Yahr & Wolfgang, 2006). Whereas the T3SS was induced in a *bvlR* mutant, the ability of the mutant to form tight microcolonies was lost. This is not surprising as the T3SS and biofilm formation are often inversely regulated (Furukawa *et al.*, 2006).

Tight microcolony formation is a configuration of biofilm formation associated with the CF lung. This mode of biofilm formation differs from typical biofilm formation as it does not require a surface to attach to. Instead, cells self-aggregate to form free-floating biofilm-like structures. Tight microcolony formation is particularly relevant to the CF lung as it has been shown that 95 % of *P. aeruginosa* found in the CF lung are present at a distance greater than 5 μm from the epithelial cell surface in non-surface-attached tight microcolonies (Worlitzsch *et al.*, 2002). The tight microcolony form of biofilm formation may have very different phenotypic and genetic properties from the classically studied surface-attached biofilm. The influence of BvlR on this phenotype is probably mediated through its role in repressing *cupA*-associated fimbrial-based surface attachment (Lizewski *et al.*, 2004; Mulcahy & Lewenza, 2011; Vallet *et al.*, 2001). Surface attachment is upregulated in a *bvlR* mutant most likely through the increased transcription of the *cupA* operon. While fimbrial attachment is key to the initial stages of biofilm formation on a surface, it has been shown to be downregulated as the biofilm formation process proceeds (Lizewski *et al.*, 2004; Mulcahy & Lewenza, 2011; Vallet *et al.*, 2001). The identification of BvlR as a regulator of this form of biofilm adds another novel gene to the complex regulatory network already involved in controlling biofilm formation. BvlR is the first LTTR to be described with a role in tight microcolony formation.

Experiments with the *C. elegans* model of infection demonstrated that the presence of BvlR reduces the capacity of *P. aeruginosa* to establish acute infection. BvlR was shown to significantly reduce *toxA* expression, one of the key virulence determinants through which *P. aeruginosa* is capable of

killing *C. elegans* (Fogle *et al.*, 2002; Matsumoto *et al.*, 1999). Furthermore, the identification of homologues of BvlR in a wide variety of respiratory pathogens may indicate a positive selection for BvlR consistent with its potential role in infection in the niche environment of the lung (Fig. S4).

BvlR is the first LTTR described in *P. aeruginosa* to function predominantly as a transcriptional repressor. BvlR fulfils a number of the 'classical' features associated with LTTRs, such as the ability to negatively auto-regulate its own expression, and to regulate expression of a divergently transcribed gene, *bvlA* (Maddocks & Oyston, 2008; Schell, 1993). LTTRs are typically associated with positive transcriptional regulation of divergent targets. BvlR exhibits an unusual mode of action in its regulation of BvlA, as it negatively affects its expression. BvlR was found to bind to the *bvlR–bvlA* intergenic region, specifically to the TCCCGAATAGAGA motif, consistent with the classic LTTR T-N_{11}-A binding box (Parsek *et al.*, 1994). Additional complete BvlR binding motifs were not identified in the *P. aeruginosa* genome sequence. This suggests that redundancy may exist with some of the nucleotides within the motif. Bioinformatic analysis identified a number of partially conserved motifs within the *P. aeruginosa* genome sequence. Of these potential binding targets, BvlR was shown to directly bind to the *sigX–oprF* intergenic region. In spite of the specific *in vitro* interaction between BvlR and this promoter region, expression was not altered in the mutant or complemented strain. This may be due to the complex nature of *oprF* transcriptional regulation, with four different promoters all having a significant impact on *oprF* transcription with varying degrees of importance depending on a range of environmental and metabolic conditions (Bouffartigues *et al.*, 2012). It is interesting to note that *oprF* is upregulated in contact with sputum, and plays a crucial role in the ability of *P. aeruginosa* to form tight microcolonies within ASM. Together, this suggests that a potential regulatory interplay may exist between *oprF* and BvlR in specific environments such as the CF lung. Outside of the evidence presented of the *bvlR–bvlR* and *sigX–oprF* intergenic regions being direct binding targets for purified BvlR, partially conserved BvlR binding motifs were also identified upstream of a number of genes that were modulated in the transcriptome, such as *plcR*, *antA* and *coxB*, suggesting these may also be BvlR direct binding targets but only in the presence of a ligand (Fig. S2). Future work will focus on distinguishing the direct and indirect downstream regulatory pathways that are influenced by BvlR.

In agreement with the data from the molecular analysis of BvlR, global transcriptome analysis highlighted the potential role of BvlR as a transcriptional repressor, as it was shown to downregulate the expression of its targets. A number of transcriptional regulators were seen to be modulated within the transcriptome dataset, although searches of the available literature did not establish a direct link between these regulators and the virulence phenotypes associated with BvlR. Note, however, that BvlR expression did repress the transcription of another predicted LTTR, PA14_27400, and that phylogenetic analysis of the evolution of LTTR proteins in *P. aeruginosa* clustered both PA2877 and PA2838 (PA14_27400) together in a single clade (Reen *et al.*, 2013b). An interesting finding in that paper was the interfacing of clustered regulatory networks, suggesting that a novel regulatory cascade may exist between these LTTRs. Future work will focus on the potentially ligand-specific interaction of BvlR with other transcriptional regulators that may underpin the virulence-associated role of BvlR in *P. aeruginosa*.

The evidence presented highlights the significant impact this previously uncharacterized regulator has in modulating the pathogenic potential of *P. aeruginosa*, significantly influencing the production of acute virulence determinants such as exotoxin A production and the T3SS. It is also shown to have a significant impact on surface attachement and a CF lung-specific mode of biofilm formation. The influence that BvlR has on *P. aeruginosa* virulence is likely to be the result of both direct and indirect effects and further work is needed to define the hierarchical pathway through which this key regulatory protein impacts on virulence. However, taken together with the previous observation of the upregulation of BvlR in response to contact with epithelial cells (Frisk *et al.*, 2004) the data suggest that BvlR is a key component of the regulatory interplay in the pathogen–host interactome.

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