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

Ronan R. McCarthy,1 Marlies J. Mooij,1,† F. Jerry Reen,1 Olivier Lesouhaitier2 and Fergal O’Gara1,3

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).
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 \textit{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 \( PA14_26880 \) as a key regulator of virulence-associated phenotypes and pathogenesis in \( P. \) aeruginosa.

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 \( ^\circ \)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 \( \mu \)g ml\(^{-1}\), tetracycline 20 \( \mu \)g ml\(^{-1}\), chloramphenicol 20 \( \mu \)g ml\(^{-1}\), \( P. \) aeruginosa – tetracycline 25 \( \mu \)g ml\(^{-1}\), gentamicin 20 \( \mu \)g ml\(^{-1}\), streptomycin 100 \( \mu \)g ml\(^{-1}\).

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<th>Name</th>
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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-Dussois, 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 pMebvIR, the PA14_26880 ORF was amplified using primers bvIR OEF and bvIR OER, which contain incorporated BglII and KpnI 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 XhoI and KpnI restriction sites, respectively. The XbaI–KpnI exsC fragment was cloned into XbaI–KpnI-digested pMP190 (Spaink et al., 1987) to produce pMP190_exsC.

To construct a plasmid expressing a His-tagged BvIR protein, the bvIR ORF was amplified using primers BvIR His F and BvIR His R, which incorporate NcoI and HindIII restriction sites, respectively. Finally, the amplicon was cloned into the MCS of pET28a to yield pET28abvIRH6C, which expressed a C-terminal His-tagged BvIR protein.

Transcriptional fusions to luxCDABE were generated by cloning the promoter regions of PA14_26880, amplified by PCR using primer sets bvIR TFF and bvIR TFR. The amplicon was flanked by Xhol–BamHI restriction sites, allowing it to be ligated into the sites Xhol–BamHI of pMS5402 (Duan & Surette, 2007). Luminescence was measured over time using a Teco 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 OD600 of 0.05 in 20 ml and incubated at 37 °C for 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 Sramiramu et al. (2005). ASM (1 ml) was inoculated to an OD600 of 0.05 with an overnight culture of P. aeruginosa PA14 in 24-well cell-culture plates (Starstedt). 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 CaCl2, 1 ml 1 M MgSO4, 25 ml 1 M KH2PO4) 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 OD600 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 OD600 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. OD600 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 H2O. 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 microliters was then taken from each well and added to a clean 96-well plate (Corning) and OD570 was measured.

Flow cell biofilm assay. Flow cell chambers with individual channel dimensions of 1 x 4 x 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 OD600 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 (OD600 of 0.7–0.8) of PA14 pME6032, PA14::bvIR pME6032 and PA14::bvIR pME6032_bvIR using the RNAeasy mini kit (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.

**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

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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 pME6032 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 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.

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.
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 pMEbvlR 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.

**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 (pET28abvlRH6C) 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-N11-A, the bvlR–bvlA intergenic region was analysed for such a motif. A single T-N11-A (TCCCGAATAGAG) 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
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. 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.

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)](https://mic.sgmjournals.org/1495)

<table>
<thead>
<tr>
<th>ORF ID</th>
<th>Gene name</th>
<th>Description</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA3541</td>
<td>alg8</td>
<td>Alginate biosynthesis protein</td>
<td>2.5</td>
</tr>
<tr>
<td>PA3548</td>
<td>algI</td>
<td>Alginate O-acetyltransferase</td>
<td>2.1</td>
</tr>
<tr>
<td>PA3549</td>
<td>algJ</td>
<td>Alginate O-acetyltransferase</td>
<td>2.0</td>
</tr>
<tr>
<td>PA3543</td>
<td>algK</td>
<td>Alginate biosynthetic protein</td>
<td>10.6</td>
</tr>
<tr>
<td>PA3547</td>
<td>algL</td>
<td>Poly(β-1-mannuronate) lyase precursor</td>
<td>2.1</td>
</tr>
<tr>
<td>PA2129</td>
<td>cupA2</td>
<td>Chaperone</td>
<td>2.2</td>
</tr>
<tr>
<td>PA2132</td>
<td>cupA5</td>
<td>Chaperone</td>
<td>2.4</td>
</tr>
<tr>
<td>PA4084</td>
<td>cupB3</td>
<td>Usher</td>
<td>7.2</td>
</tr>
<tr>
<td>PA4083</td>
<td>cupB4</td>
<td>Chaperone</td>
<td>2.6</td>
</tr>
<tr>
<td>PA4082</td>
<td>cupB5</td>
<td>Adhesive protein</td>
<td>4.1</td>
</tr>
<tr>
<td>PA4081</td>
<td>cupB6</td>
<td>Fimbrial subunit</td>
<td>3.2</td>
</tr>
<tr>
<td>PA2235</td>
<td>pslE</td>
<td>Exopolysaccharide biosynthesis</td>
<td>2.3</td>
</tr>
<tr>
<td>PA2236</td>
<td>pslF</td>
<td>Exopolysaccharide biosynthesis</td>
<td>2.4</td>
</tr>
</tbody>
</table>

**Genes associated with attachment and biofilm**

<table>
<thead>
<tr>
<th>ORF ID</th>
<th>Gene name</th>
<th>Description</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA4813</td>
<td>lipC</td>
<td>Lipase</td>
<td>8.4</td>
</tr>
<tr>
<td>PA3319</td>
<td>plcN</td>
<td>Non-haemolytic phospholipase C precursor</td>
<td>2.6</td>
</tr>
<tr>
<td>PAO843</td>
<td>plcR</td>
<td>Phospholipase accessory protein</td>
<td>2.0</td>
</tr>
<tr>
<td>PA1148</td>
<td>toxA</td>
<td>Exotoxin A precursor</td>
<td>2.4</td>
</tr>
<tr>
<td>PAO707</td>
<td>toxR</td>
<td>Transcriptional regulator</td>
<td>2.4</td>
</tr>
</tbody>
</table>
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
lungs, 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 pME6bI 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 fulfills 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-N11-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 attachment 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 (Frisik et al., 2004) the data suggest that BvlR is a key component of the regulatory interplay in the pathogen–host interactome.

ACKNOWLEDGEMENTS

This research was supported in part by grants awarded by the Science Foundation of Ireland (07/IN.1/B948, 12/TIDA/B2411, 12/TIDA/B2405, 09/RFP/BMT 2350, SSPC212/RC/2275); the Department of Agriculture, Fisheries and Food (FIRM/RSF/CoForRD; DAFF11/F/009MabS); the Environmental Protection Agency (EPA 2008-PhD/S-2); the Irish Research Council for Science, Engineering and Technology (PD/2011/2414; RS/2010/2413); the European Commission (FP7-PEOPLE-2013-ITN, 607786; FP7-KBBE-2012-6, FP7-KBBE-2012-6, Marie Curie 256596); Marine Microbial Biodiversity, Bioinformatics and Biotechnology (MICRO B3) (OCEAN 2012 287589), Marine Micro-organisms: Cultivation Methods for improving their Biotechnological Applications (MACUMBA) (CP-TP 311975), Increasing Value and Flow in the Marine Biodiscovery Pipeline (PharmaSea) (CP-TP 312184) and the Marine Institute (Beadfort award C2cRA 2007/082); Teagasc (Walsh Fellowship 2013) and the Health Research Board (HRA/2009/146).

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\textbf{Edited by: J. Kok}