Low oxygen induces the type III secretion system in *Pseudomonas aeruginosa* via modulation of the small RNAs *rsmZ* and *rsmY*

Julie O'Callaghan, F. Jerry Reen, Claire Adams and Fergal O'Gara

BIOMERIT Research Centre, Department of Microbiology, University College Cork, Cork, Ireland

Correspondence
Fergal O'Gara
f.ogara@ucc.ie

Received 14 June 2011
Revised 22 July 2011
Accepted 20 August 2011

A steep oxygen gradient within the mucus of the cystic fibrosis (CF) lung combined with the biofilm mode of bacterial growth forces respiratory pathogens to adapt to varying oxygen availability. This study presents the novel finding that the *Pseudomonas aeruginosa* response to limiting oxygen stress includes induction of its type III secretion system (T3SS), which subsequently contributes towards host cell cytotoxicity. In *P. aeruginosa*, the global anaerobic response regulator Anr perceives low oxygen and subsequently triggers gene expression of a range of target genes, including the response regulator *narL*. Here we demonstrate that microaerobic induction of the T3SS is dependent on Anr, and that this is mediated through direct NarL transcriptional repression of the sRNAs *rsmY* and *rsmZ*, allowing free RsmA protein to positively regulate the T3SS. This study reveals a novel interplay between the Anr–NarL and RsmAYZ regulatory circuits, and introduces RsmA as an important regulator during *P. aeruginosa* adaptation to a low-oxygen environment.

INTRODUCTION

The human pathogen *Pseudomonas aeruginosa* is a highly versatile and important nosocomial pathogen, and is the main cause of chronic lung infections in cystic fibrosis (CF) patients. The pathogenicity of *P. aeruginosa* is attributed to an arsenal of virulence-associated phenotypes, including the type III secretion system (T3SS), through which effector toxins are injected directly into host cells, leading to inhibition of phagocytosis and increased cytotoxicity (Cowell *et al.*, 2005; Garrity-Ryan *et al.*, 2000; Geiser *et al.*, 2001). The T3SS, which is induced by human cells and in low calcium *in vitro*, is tightly controlled via complex molecular networks (Hauser, 2009). Recently, the global post-transcriptional regulator RsmA has been implicated in the regulation of the T3SS (Mulcahy *et al.*, 2006). RsmA activity is itself modulated by the two small, non-coding RNAs *rsmY* and *rsmZ*, which sequester and thus inactivate the protein (Kay *et al.*, 2006). *rsmY* and *rsmZ* transcription is regulated by the response regulator GacA, and in the case of *rsmZ* may involve the DNA-binding protein integration host factor (IHF) (Humair *et al.*, 2010).

In CF patients, mutation(s) in the cystic fibrosis transmembrane conductance regulator (CFTR) results in thickened mucus formation in the respiratory airway and leads to

reduced oxygen availability deep within the lungs (Alvarez-Ortega & Harwood, 2007; Boucher, 2004; Matsui *et al.*, 2006; Worlitzsch *et al.*, 2002; Yoon *et al.*, 2002). Long-term persistence of *P. aeruginosa* in the CF lung is aided by its ability to reside in the dense hyper-mucoid lumen as hypoxic biofilm-like masses, leading to chronic infections refractory to the host immune response and antibiotic treatment (Alvarez-Ortega & Harwood, 2007; Matsui *et al.*, 2006; Stewart & Franklin, 2008; Worlitzsch *et al.*, 2002). Several studies have demonstrated that microaerobic to anaerobic conditions dominate in *P. aeruginosa* biofilms (Hassett *et al.*, 2002; Stewart & Franklin, 2008; Worlitzsch *et al.*, 2002; Yoon *et al.*, 2002). Thus, CF-associated pathogens may only survive and persist in the infected lung by adapting to limiting oxygen stress. *P. aeruginosa* perceives and responds to low oxygen stress via the global anaerobic stress response regulator Anr (Sawers, 1991; Trunk *et al.*, 2010; Zimmermann *et al.*, 1991). Anr is a homologue of the *Escherichia coli* anaerobic response master regulator FNR, and it functions by regulating a large number of genes required for anaerobic respiration, including *dnr* and the *narXL* two-component system (Sawers, 1991; Trunk *et al.*, 2010; Zimmermann *et al.*, 1991). During low-oxygen stress, the NarL response regulator is involved in coordinating energy metabolism through induction of nitrate metabolism (Palmer *et al.*, 2007; Schreiber *et al.*, 2007; Sharma *et al.*, 2006) and repression of less efficient modes of energy generation such as pyruvate and arginine fermentation (Benkert *et al.*, 2008).

Recent reports have described the contrasting effects of oxygen limitation on the T3SS of two bacterial pathogens,

Abbreviations: CF, cystic fibrosis; EMSA, electrophoretic mobility shift assay; IHF, integration host factor; RLC, relative light counts; T3SS, type III secretion system.

Two supplementary figures and a supplementary table are available with the online version of this paper.

E. coli and *Shigella flexneri* (Ando *et al.*, 2007; Marteyn *et al.*, 2010; Schüller & Phillips, 2010). However, the important question of whether oxygen availability influences the T3SS in *P. aeruginosa* remains to be addressed. While transcriptome analyses of the molecular response of *P. aeruginosa* to low oxygen have not yet identified any alteration in T3SS-associated gene expression (Alvarez-Ortega & Harwood, 2007; Trunk *et al.*, 2010), T3SS-associated gene expression is induced in anaerobic biofilms (Waite & Curtis, 2009).

In this study we demonstrate that the T3SS is activated specifically in response to low oxygen tension and that the regulatory mechanism of this activation involves an interplay between the oxygen-sensing Anr–NarL and the RsmAYZ regulatory circuits. We demonstrate that the expression of the two small RNAs *rsmY* and *rsmZ* is significantly suppressed by microaerobic conditions, and this effect is mediated via the Anr-regulated response regulator NarL.

METHODS

Bacterial strains and growth conditions. The *P. aeruginosa* strains used in this study are shown in Table 1. Bacteria were routinely grown at 37 °C in Tryptic Soy Broth (TSB) medium supplemented with 1 % KNO₃ as the nitrogen source. A gentamicin-resistant transposon insertion mutant of the *anr* gene was obtained from the PA14 Transposon Insertion Mutant Library (Liberati *et al.*, 2006), while a tetracycline-resistant transposon insertion mutant of the *narL* gene was obtained from the mPAO1 Transposon Insertion Mutant Library (Jacobs *et al.*, 2003). Transposon type, insertion location and orientation were confirmed for the mutants. Type III-inducing (low Ca²⁺) and non-inducing media (high Ca²⁺) were prepared by addition of 5 mM EGTA and 5 mM CaCl₂ to TSB, respectively (Wolfgang *et al.*, 2003). The 10 ml aerobic cultures were grown in 70 ml Wheaton serum flasks at 37 °C and 160 r.p.m. Anaerobic cultures were grown similarly, but the oxygen was replaced with oxygen-free nitrogen gas using sealed Wheaton serum flasks. Anaerocult A (Merck) packs were used to generate microaerobic conditions for 35 ml cultures grown in 35 ml Wheaton flasks at 37 °C and 160 r.p.m. Anaerocult A has been reported to generate 0.5 % O₂ concentrations within approximately 90 min (Imhof & Heinzer, 1996). Cells were harvested from bacterial cultures in all three oxygen conditions during the mid-exponential phase of growth at OD₆₀₀ 0.5.

E. coli strains were routinely grown in TSB media at 37 °C. Where appropriate, antibiotics were added to growth media at the following concentrations: 50 µg tetracycline ml⁻¹, 15 µg gentamicin ml⁻¹, 100 µg kanamycin ml⁻¹ and 200 µg streptomycin ml⁻¹ for *P. aeruginosa*; and 50 µg ampicillin ml⁻¹, 5 µg gentamicin ml⁻¹, 10 µg tetracycline ml⁻¹, 40 µg kanamycin ml⁻¹ and 100 µg streptomycin ml⁻¹ for *E. coli*.

Strain construction. The sequences of promoter regions in transcriptional reporter fusions provided by other laboratories were determined and confirmed to be identical to that of the PAO1 sequence (<http://www.pseudomonas.com/>). Promoter regions used for the construction of transcriptional reporter fusions were amplified using primer pairs based on the PAO1 and PA14 genome sequences (<http://www.pseudomonas.com/>) (Supplementary Table S1), and subcloned into pmini-CTX-*lux* (Becher & Schweizer, 2000). Plasmids derived from pmini-CTX-*lux* and pMP190 were mobilized into PAO1, mPAO1, PA14 and mutant strains by triparental mating. After integration of pmini-CTX-*lux* into the *attB* site, vector backbone sequences were excised by Flp recombinase as described elsewhere

(Hoang *et al.*, 1998). Each reporter construct was therefore present as a single, unmarked copy integrated at the chromosomal *attB* site.

To generate complementation and overexpression constructs, fragments containing *anr* and *narL* genes were subcloned into pBBR1-MCS2 (Kovach *et al.*, 1995) and mobilized by triparental mating into their respective mutants and wild-types.

Measurement of luciferase/β-galactosidase and calculation of transcription. Bacteria were cultured under microaerobic and aerobic growth conditions as described above, in low and high Ca²⁺-conditioned TSB media. At OD₆₀₀ 0.5, relative light counts (RLC) were compared between strains carrying the promoter::*lux* versus empty::*lux* fusions using a Tecan GENios plate reader (Tecan Group). At OD₆₀₀ 0.5, β-galactosidase activity was compared between strains carrying the promoter::*lacZ* versus empty::*lacZ* fusions using a protocol derived from Zhang & Bremer (1995). Bacteria were subjected to serial dilutions and plating to confirm uniform growth between wild-type and mutant strains.

Immunoblotting of type III proteins. Whole-cell protein samples were prepared and immunoblotting was performed as described by Hauser *et al.* (2002). Briefly, PAO1 was cultured as described above, and following isolation, proteins were subjected to SDS-PAGE (15 µg protein per lane) and transferred onto nitrocellulose membranes. Membranes were probed with rabbit antisera against ExoS, ExoT, PopB, PopD and PcrV [kind gifts from Ina Attree (CEA, Grenoble, France) and Alan Hauser (Northwestern University, Chicago, IL, USA)], followed by secondary goat anti-rabbit IgG conjugated with horseradish peroxidase (DakoCytomation) and developed with chemiluminescent substrate (Bio-Rad). Exposed films were scanned and images processed using Adobe Photoshop 6.0.

In silico analysis of *rsmYZ* and *anr* promoter regions. Prediction of transcriptional factor binding sites for the promoter regions of *rsmA*, *rsmY* and *rsmZ* were made using Virtual Footprint, which recognizes single or composite DNA patterns (http://www.prodoric.de/vfp/vfp_promoter.php), or the PromScan program, which allows scanning for ungapped sequence motifs (<http://molbiol-tools.ca/promscan/>). The transcriptional factor binding sites detected using these software packages were then compared with the published consensus sequence for specific binding motifs.

NarL–His₁₂ purification and in vitro phosphorylation. The *narL* gene from *P. aeruginosa* PAO1 was amplified using gene-specific primers (Supplementary Table S1) and cloned into the pET28a inducible expression plasmid (Invitrogen). Constructs were transformed into *E. coli* BL21 Codon Plus BL21(DE3) cells, giving rise to NarL fused to both N-terminal and C-terminal His₆ tags. Overnight Luria broth (LB) cultures were transferred into fresh media supplemented with appropriate antibiotics and grown to OD₆₀₀ 0.8, at which time IPTG was added to a final concentration of 0.7 mM. The cultures were grown for a further 4 h, and cells were harvested at 4 °C following centrifugation at 10 000 r.p.m. for 10 min. The supernatants were removed and cells stored overnight at –80 °C to enhance lysis. Cell pellets were resuspended in Cell Lytic B (Sigma-Aldrich) and lysozyme (Sigma-Aldrich), benzonase (Sigma-Aldrich) and protease inhibitors (Sigma-Aldrich) were added to the cells, which were subsequently incubated at room temperature for 15 min with shaking (100 r.p.m.). Following centrifugation at 4 °C, the clarified supernatant was purified on a Poly-Prep chromatography column (Bio-Rad) containing HIS-Select Nickel Affinity Gel (Sigma-Aldrich) and eluted using increasing concentrations of imidazole. Fractions were collected and analysed on SDS-PAGE gels to identify the fraction containing purified NarL protein. The concentration of purified NarL–His₁₂ protein was determined by Bradford assay. NarL (10 µg) underwent *in vitro* phosphorylation at room temperature for 1 h in 100 µl freshly

Table 1. Strains and plasmids used in this study

Abbreviations: Amp^r, ampicillin resistant; Gm^r, gentamicin resistant; Km^r, kanamycin resistant; St^r, streptomycin resistant; Tc^r, tetracycline resistant.

Strain or plasmid	Description	Source or reference
<i>P. aeruginosa</i> strains		
PAO1	Wild-type	Holloway collection
$\Delta rsmA$	<i>rsmA</i> deletion mutant of PAO1	Mooij <i>et al.</i> (2010)
$\Delta rsmA$ comp	$\Delta rsmA$ pME6032:: <i>rsmA</i> ; Tc ^r	Mooij <i>et al.</i> (2010)
PA14	Wild-type	Liberati <i>et al.</i> (2006)
PA14 <i>anr</i>	PA14 <i>anr</i> ::Tn; Gm ^r	This study
PA14 <i>anr</i> comp	PA14 <i>anr</i> ::Tn; pBBR1MCS2:: <i>anr</i> ; Gm ^r ; Km ^r	This study
PA14 <i>anr</i> pBBR: <i>narL</i>	PA14 <i>anr</i> ::Tn; pBBR1MCS2:: <i>narL</i> ; Gm ^r ; Km ^r	This study
mPAO1	Wild-type	Jacobs <i>et al.</i> (2003)
mPAO1 <i>narL</i>	mPAO1 <i>narL</i> ::Tn; Tc ^r	Jacobs <i>et al.</i> (2003)
mPAO1 <i>narL</i> comp	mPAO1 <i>narL</i> ::Tn; pBBR1MCS2:: <i>narL</i> ; Tc ^r ; Km ^r	This study
Plasmids		
pPS858	FRT; Amp ^r	Hoang <i>et al.</i> (1998)
pRK2013	Helper vector; Km ^r	Figurski & Helinski (1979)
mini-CTX-lux	Vector used for construction of transcriptional fusions with the <i>luxCDABE</i> operon from <i>Xenorhabdus luminescens</i> ; contains <i>attP</i> site for integration at the <i>attB</i> site of <i>P. aeruginosa</i> chromosome; Tc ^r	Becher & Schweizer (2000)
BBR ₁ -MCS2	Broad-host-range cloning vector; Km ^r	Kovach <i>et al.</i> (1995)
MP190	Vector used for construction of transcriptional fusions with the <i>lacZ</i> gene; St ^r	Spaink <i>et al.</i> (1987)
pET28a	T7 promoter-driven His-tag protein expression vector; Km ^r	Novagen
mini-CTX- <i>exoS</i> -lux	303 bp <i>EcoRI</i> – <i>Bam</i> HI PCR-amplified fragment of <i>exoS</i> promoter region in <i>EcoRI</i> – <i>Bam</i> HI of mini-CTX-lux; Tc ^r	Laskowski <i>et al.</i> (2004)
mini-CTX- <i>exoU</i> -lux	310 bp <i>EcoRI</i> – <i>Bam</i> HI PCR-amplified fragment of <i>exoU</i> promoter region in <i>EcoRI</i> – <i>Bam</i> HI of mini-CTX-lux; Tc ^r	Laskowski <i>et al.</i> (2004)
mini-CTX- <i>exsC</i> -lux	300 bp <i>EcoRI</i> – <i>Bam</i> HI PCR-amplified fragment of <i>exsC</i> operon promoter region in <i>EcoRI</i> – <i>Bam</i> HI of mini-CTX-lux; Tc ^r	Laskowski <i>et al.</i> (2004)
mini-CTX- <i>rsmA</i> -lux	224 bp <i>XhoI</i> – <i>SalI</i> PCR-amplified fragment of <i>rsmA</i> operon promoter region in <i>XhoI</i> – <i>SalI</i> of mini-CTX-lux; Tc ^r	This study
mini-CTX- <i>rsmY</i> -lux	328 bp <i>XhoI</i> – <i>SalI</i> PCR-amplified fragment of <i>rsmY</i> operon promoter region in <i>XhoI</i> – <i>SalI</i> of mini-CTX-lux; Tc ^r	This study
mini-CTX- <i>rsmZ</i> -lux	328 bp <i>XhoI</i> – <i>SalI</i> PCR-amplified fragment of <i>rsmZ</i> operon promoter region in <i>XhoI</i> – <i>SalI</i> of mini-CTX-lux; Tc ^r	This study
mini- <i>exoS</i> - <i>lacZ</i>	<i>exoS</i> – <i>lacZ</i> transcriptional fusion in pUC18miniTn7T; Gm ^r	Tian <i>et al.</i> (2009)
MP190- <i>rsmZ</i> - <i>lacZ</i>	<i>rsmZ</i> – <i>lacZ</i> transcriptional fusion in pMP190; St ^r	This study
BBR ₁ -MCS2:: <i>anr</i>	943 bp <i>KpnI</i> – <i>XhoI</i> PCR-amplified fragment of <i>anr</i> gene in <i>KpnI</i> – <i>XhoI</i> of BBR ₁ -MCS2; Km ^r	This study
BBR ₁ -MCS2:: <i>narL</i>	722 bp <i>EcoRI</i> – <i>SacI</i> PCR-amplified fragment of <i>narL</i> gene in <i>EcoRI</i> – <i>SacI</i> of BBR ₁ -MCS2; Km ^r	This study
pET28a:: <i>narL</i> -His ₆	659 bp PCR-amplified fragment of <i>narL</i> gene in pET28a; Km ^r	This study

made 100 mM Tris/HCl (pH 7.0), 10 mM MgCl₂, 125 mM KCl and 50 mM lithium acetyl phosphate (Sigma-Aldrich).

Electrophoretic mobility shift assay (EMSA). Target promoter DNA corresponding to fragments of the *rsmY*, *rsmZ*, *narK* and *phoB* promoter regions was amplified using infrared dye end-labelled promoter-specific oligonucleotides (Supplementary Table S1). These fragments were purified with a Qiagen gel extraction kit. Protein–DNA binding assays containing 15 fmol DNA and 0.5 µg ml^{−1} poly dIdC (Roche) with increasing concentrations of NarL protein were performed at room temperature for 30 min in Roche 5 × binding buffer (Roche). After completion of the binding assay, 4 µl 50 % (v/v) glycerol/Orange G buffer was added to the samples, which were subsequently analysed on a 6 % polyacrylamide gel in 0.5 × TBE buffer run at 90 V for 1 h. Gels were visualized using an Odyssey Imager (LI-COR Biosciences).

Statistical analysis. PAO1, mPAO1 and PA14 microaerobic and aerobic promoter expression was analysed using Student's *t* test. All analysis was carried out using the SPSS and Excel software packages. A *P* value of 0.05 or less was considered significant.

RESULTS

T3SS is induced by low oxygen tension

The T3SS in *P. aeruginosa* has been shown to be induced by host cells and under low-calcium conditions aerobically. However, as limited oxygen availability has been reported to be a physiologically relevant stress in the CF lung, we

Fig. 1. The *P. aeruginosa* PAO1 T3SS can be activated by oxygen-limiting stress via the RsmAYZ regulatory network. (a) Luminescence produced by PAO1 carrying a single copy of the (i) *exsC::lux* or (ii) *exoS::lux* transcriptional reporter was measured during aerobic (black bars) and microaerobic (white bars) growth in TSB/1 % KNO₃ with high and low Ca²⁺ to OD₆₀₀ 0.5. (iii) PAO1 was cultured in TSB/1 % KNO₃ with high and low Ca²⁺ to OD₆₀₀ 0.5 under anaerobic, microaerobic and aerobic growth conditions. Whole-cell proteins (15 µg) were prepared and separated by SDS-PAGE. Membranes were probed with antisera against ExoT, ExoS, PopB, PopD and PcrV. Detected proteins and molecular mass (kDa; in parentheses) are indicated. (b) (i) β -Galactosidase activity produced by PAO1 (black bars), Δ rsmA (white bars) and Δ rsmA complemented (hatched bars) carrying the *exoS::lacZ* transcriptional reporter were compared during aerobic and microaerobic conditions in TSB/1 % KNO₃ with high and low Ca²⁺ at OD₆₀₀ 0.5. Luminescence produced by PAO1 carrying a single copy of the (ii) *rsmY::lux* or (iii) *rsmZ::lux* transcriptional reporter was compared during aerobic (black bars) and microaerobic (white bars) growth in TSB/1 % KNO₃ with high and low Ca²⁺ at OD₆₀₀ 0.5. For reporter activity experiments, results are presented as β -galactosidase activity or RLC compared with promoterless constructs. Data represent triplicate samples from three biological replicate experiments; error bars, SDs for triplicate experiments. Asterisks indicate $P \leq 0.05$ for aerobic versus microaerobic conditions (Student's *t* test).

sought to determine whether low oxygen influenced expression of the T3SS in *P. aeruginosa*. *exsC* and *exoS* transcriptional promoter fusions were used as indicators of the T3SS, and activity was assessed in *P. aeruginosa* PAO1 in response to oxygen availability. During microaerobic growth, *exsC* and *exoS* promoter activity was triggered in both inducing (low Ca²⁺) and non-inducing (high Ca²⁺) conditions; this was in contrast to aerobic growth, where high expression was only seen using inducing conditions of low Ca²⁺ (Fig. 1a, i, ii). These results suggested that limiting oxygen may in fact activate the T3SS.

To determine the extent of T3SS induction in response to limiting oxygen, the effect of varying oxygen availability on levels of the type III effector toxin and secretion machinery proteins ExoT, ExoS, PopB, PopD and PcrV was assessed by Western blot analysis. PAO1 was cultured in low and high Ca²⁺ using anaerobic, microaerobic and aerobic growth conditions. All proteins were detected when grown anaerobically and microaerobically, independent of Ca²⁺ concentrations, whereas proteins were only detected when grown aerobically in inducing conditions of low Ca²⁺ (Fig. 1a, iii). Interestingly, induction of the T3SS was stronger under anaerobic conditions relative to microaerobic conditions, suggesting that oxygen concentration is a critical factor in this response. Under anaerobic conditions, there was a slight difference in the quantity of ExoS/T protein detected in the presence of high Ca²⁺ compared with low Ca²⁺. Although all cultures were harvested at OD₆₀₀ 0.5, strains grew marginally better in the presence of high Ca²⁺; however, the growth difference was not significant. We believe that this is the first evidence of elevated type III exoprotein and machinery production in *P. aeruginosa* cultured under oxygen-limiting conditions, which implicates oxygen availability as an important environmental condition modulating the T3SS in *P. aeruginosa* PAO1.

T3SS induction in response to low oxygen is modulated via RsmAYZ

Recently we established that RsmA plays a role in the positive regulation of the *P. aeruginosa* T3SS in response to host cells (Mulcahy *et al.*, 2006), and therefore sought to

investigate whether this network also modulates the T3SS response to limiting oxygen. Initially, an *rsmA* mutant was used to assess *exoS* expression under microaerobic conditions. Unlike PAO1, the *rsmA* mutant failed to induce *exoS* expression under microaerobic conditions independently of Ca²⁺ concentration, and also under low Ca²⁺ in the presence of oxygen (Fig. 1b, i). Thus, RsmA has a regulatory role in T3SS modulation during both aerobic and microaerobic growth.

The small non-coding RNAs *rsmY* and *rsmZ* sequester and titrate RsmA to dictate the critical balance of this regulatory protein within the bacterial cell (Pessi *et al.*, 2001). Conversely, RsmA itself can also modulate sRNA production (Kay *et al.*, 2006). Therefore, to assess the level at which low oxygen influences the RsmA/Y/Z network, *rsmA*, *rsmY* and *rsmZ* promoter activity was examined in PAO1 cultured microaerobically and aerobically in low and high Ca²⁺. In agreement with the T3SS experiments, *rsmY* and *rsmZ* expression was downregulated by microaerobic conditions independently of Ca²⁺ concentration, and also by low Ca²⁺ in the presence of oxygen (Fig. 1b, ii, iii). Aerobic expression of these small RNAs was higher in high-Ca²⁺ media (non-inducing). In contrast, the transcription of *rsmA* was similar under all conditions tested (data not shown). This suggests that low oxygen may trigger induction of the T3SS through suppression of *rsmYZ* expression, which leads to increased RsmA activity. Interestingly, the data also suggest that modulation of *rsmY* and *rsmZ* plays a part in low-Ca²⁺ induction of the T3SS, which to the best of our knowledge has not been demonstrated to date.

Anr regulates expression of *rsmYZ* in response to low oxygen tension

P. aeruginosa perceives low oxygen tension through the anaerobic regulator Anr, a homologue of *E. coli* FNR, documented to be the general positive activator of the anaerobic response (Sawers, 1991; Trunk *et al.*, 2010; Zimmermann *et al.*, 1991). Therefore, using a *P. aeruginosa* PA14 *anr* mutant (Liberati *et al.*, 2006), we investigated whether microaerobic induction of the T3SS was

Fig. 2. Anr and NarL positively regulate the T3SS response to oxygen depletion through modulating *rsmY* and *rsmZ*. (a) Luminescence produced by PA14 (black bars), the *anr* mutant (white bars) or the complemented *anr* mutant (hatched bars) carrying a single copy of the (i) *exoU::lux*, (ii) *rsmY::lux* or (iii) *rsmZ::lux* transcriptional reporter was compared during aerobic and microaerobic growth in TSB/1 % KNO₃ with high and low Ca²⁺ at OD₆₀₀ 0.5. (b) Luminescence produced by PA14 (black bars), PA14pBBR::*narL* (stippled bars), the *anr* mutant (white bars) or *anr*pBBR::*narL* (hatched bars) carrying a single copy of the (i) *exoU::lux*, (ii) *rsmY::lux* or (iii) *rsmZ::lux* transcriptional reporter was compared during aerobic and microaerobic growth in TSB/1 % KNO₃ with high and low Ca²⁺ at OD₆₀₀ 0.5. Results are presented as RLC compared with promoterless constructs. Data from triplicate samples from three biological replicate experiments are presented; error bars, SDs for triplicate experiments. Asterisks indicate $P \leq 0.05$ for PA14 versus the *anr* mutant (Student's *t* test).

Anr-dependent and whether this occurred through perturbation of *rsmYZ* expression. It has been previously documented that *anr* mutants fail to grow anaerobically (Zimmermann *et al.*, 1991), and therefore *exoU* and *rsmA/Y/Z* promoter activity was compared between PA14 and the PA14 *anr* mutant during microaerobic and aerobic growth in inducing (low Ca²⁺) and non-inducing (high Ca²⁺) media.

In agreement with our PAO1 results, PA14 *exoU* promoter activity was induced during microaerobic growth, irrespective of the Ca²⁺ concentration of the medium, and was only induced during aerobic growth under low Ca²⁺ (Fig. 2a, i). In contrast, the microaerobic induction of *exoU* promoter activity was abolished in the *anr* mutant relative to PA14 wild-type (Fig. 2a, i), indicative of a role for Anr in T3SS modulation during low oxygen tension. Interestingly, the induction of *exoU* activity by low Ca²⁺ under aerobic conditions was also significantly reduced in the *anr* mutant, suggesting a possible role for Anr in the aerobic induction of the T3SS. Furthermore, the expression of both *rsmY* and *rsmZ* remained elevated in the *anr* mutant, while it was significantly reduced in PA14 during microaerobic growth and under aerobic growth in low Ca²⁺ (Fig. 2a, ii, iii). All phenotypes were fully complemented when the *anr* gene was introduced *in trans* into the PA14 *anr* mutant. Therefore, these data suggest that low oxygen tension induces the T3SS via Anr modulation of *rsmYZ* transcription.

The Anr-dependent transcriptional factor NarL modulates *rsmY* and *rsmZ* transcription

To determine the mechanism through which Anr modulates *rsmYZ* transcription, *in silico* analysis of the *rsmAYZ* promoter regions was conducted to identify consensus sequences for oxygen-dependent regulators. Specifically, potential binding sites for the regulatory protein NarL (Tyson *et al.*, 1993), a transcriptional factor directly regulated by Anr, were located on the non-coding strand at -79 and -18 bases relative to the *rsmY* transcriptional start site and at -188, -162, -115, -93 and -19 bases relative to the *rsmZ* transcriptional start site (Supplementary Fig. S1). An additional NarL consensus sequence was identified on the coding strand of the *rsmZ* promoter at position -9. A consensus sequence for the DNA-bending protein IHF (Swinger & Rice, 2004) was located on the coding strand at 56 and 113 bases upstream of the

rsmY and *rsmZ* translational start sites, respectively (<http://www.pseudomonas.com/>). Overlapping binding sites for NarL and IHF were detected within both the *rsmY* and *rsmZ* promoters, but not the *rsmA* promoter. While NarL is mainly an activator of target genes (Schreiber *et al.*, 2007), the position of the proposed NarL boxes on the opposite strand is similar to the findings of Benkert *et al.* (2008), whereby NarL represses ArgR-mediated arginine-dependent activation of the *arcDABC* operon, and this prompted us to investigate whether NarL plays a role in low oxygen-dependent activation of the T3SS and whether this is mediated by modulation of *rsmY/Z* transcription.

To test this hypothesis, the effect of *narL* overexpression on *exoU* and *rsmY/Z* promoter activity was investigated in the *P. aeruginosa* PA14 background. Under microaerobic conditions, the overexpression of *narL* induced *exoU* promoter activity in the *anr* mutant independently of medium Ca²⁺ concentration (Fig. 2b, i). Interestingly, overexpressing *narL* had a similar effect aerobically, whereby *exoU* expression was induced in both the wild-type and the *anr* mutant under both low and high Ca²⁺ concentrations (Fig. 2b, i). Conversely, *narL* overexpression resulted in downregulation of *rsmY* and *rsmZ* promoter activity in PA14 and the PA14 *anr* mutant, again irrespective of media Ca²⁺ concentration and oxygen availability, respectively (Fig. 2b, ii, iii). Therefore, it appears that overexpressing *narL* downregulated *rsmYZ* transcription, resulting in induction of the T3SS in wild-type PA14 and the *anr* mutant. To further verify NarL downregulation of the *rsmYZ* system, the effect of *narL* mutation on *rsmZ* promoter activity was investigated using a *P. aeruginosa* mPAO1 *narL* mutant (Jacobs *et al.*, 2003). As predicted, the mutant had increased *rsmZ* promoter activity compared with mPAO1 during microaerobic growth and under aerobic and low-Ca²⁺ conditions (data not shown), providing further evidence that NarL regulates the T3SS through modulation of *rsmYZ* transcription.

NarL binds specifically to the *rsmY* and *rsmZ* promoters

To determine whether the influence of NarL on *rsmY* and *rsmZ* expression was the result of direct binding of the response regulator to these promoters, we undertook EMSA analysis. Infrared dye-labelled primers were

designed to amplify the -178 to $+38$ and -172 to $+17$ regions of the *rsmY* and *rsmZ* promoters, respectively. Purification of the NarL protein was achieved with protein fractions eluted using imidazole concentrations in the 200–500 mM concentration range. Both phosphorylated and unphosphorylated NarL protein was found to bind the *rsmY* and *rsmZ* promoters at 1 and 1.7 μM concentrations, respectively (Fig. 3 and Supplementary Fig. S2). The -258 to -88 region of the *narK* promoter, previously identified as a positive target of NarL, was shifted by both micromolar and nanomolar concentrations of phosphorylated and unphosphorylated NarL (Fig. 3 and data not shown), while the -91 to -10 region of the PhoB promoter, which does not contain a putative NarL consensus sequence, was not bound by the NarL protein (Fig. 3). Addition of equimolar concentrations of non-target PhoB promoter DNA to the *rsmY* and *rsmZ* promoter interactions did not influence the binding of NarL (Supplementary Fig. S2). Taken together, these findings indicate that the shifts observed for *rsmY* and *rsmZ* are the result of specific interactions (Fig. 3). Interestingly, the *arcD* promoter (-211 to -14) was also shifted in the presence of micromolar concentrations of NarL protein (data not shown). This suggests that NarL represses *rsmY* and *rsmZ* promoter activity by directly binding one or more of the consensus sequences described in Supplementary Fig. S1 and interfering with the transcriptional machinery required to activate expression.

DISCUSSION

This study presents the novel finding that the *P. aeruginosa* T3SS is induced in response to low oxygen and that this

induction is dependent on the anaerobic response regulator Anr. This is believed to be the first indication that oxygen limitation may influence this virulence factor, and supports recent evidence that the T3SS of *P. aeruginosa* is induced in biofilms and in artificial sputum media (Fung *et al.*, 2010; Waite & Curtis, 2009), both of which provide microaerobic growth conditions. It remains to be established why earlier *anr* mutant and microaerobic comparative transcriptome studies did not identify T3SS-related genes (Alvarez-Ortega & Harwood, 2007; Trunk *et al.*, 2010). One possibility is that they are incomplete datasets, which is supported by the fact that only one gene is altered in common between the studies. A second possibility is that experimental conditions, and therefore an additional factor, may be involved in the T3SS induction. Nevertheless, a role for the anaerobic response in activation of the T3SS is also evident in other bacterial species. In the intracellular pathogen *Salmonella enterica* serovar Typhimurium, mutation of the *anr* homologue *fnr* has been found to result in reduced expression of several genes in the *Salmonella* pathogenicity island (SPI)-1/T3SS (Fink *et al.*, 2007). The T3SS of haemorrhagic *E. coli* has also been shown to be activated by low oxygen concentrations (Ando *et al.*, 2007; Schüller & Phillips, 2010). Conversely, low oxygen has been found to inhibit the T3SS of *S. flexneri* in an FNR-dependent manner (Ando *et al.*, 2007; Marteyn *et al.*, 2010; Schüller & Phillips, 2010). Thus, while low oxygen appears to be an important factor in modulating the T3SS in distinct species, the mechanism of regulation may differ.

Here we provide evidence that in *P. aeruginosa*, Anr exerts its effect on the T3SS indirectly through the Anr-regulated response regulator NarL, which represses the transcription

Fig. 3. Phosphorylated NarL protein specifically binds to the *rsmY* and *rsmZ* promoters. Purified NarL protein forms a complex with the *narK*, *rsmY* and *rsmZ* promoter regions, each of which contains several NarL consensus sequences. Addition of NarL protein to the *phoB* promoter region did not result in an observed shift, suggesting that binding to the *narK*, *rsmY* and *rsmZ* promoters is specific. The position of the shifted band was equivalent for the three promoters (arrow), while the signal strength was greatest at the higher protein concentration. Each assay was performed in the absence of protein (Φ), and in the presence of 1 and 1.7 μM *in vitro*-phosphorylated NarL protein.

of *rsmY* and *rsmZ*, leading to an accumulation of free RsmA pools within the bacterial cell (see Fig. 4 for a proposed model). *In silico* analysis revealed possible NarL-binding sites within both the *rsmY* promoter and the *rsmZ* promoter, suggesting that NarL may prevent transcription of *rsmY* and *rsmZ* through steric hindrance of GacA and/or IHF. The presence of a NarL consensus sequence opposite to and overlapping the IHF box is consistent with an earlier finding that NarL represses *arcDABC* expression through interference with IHF binding (Benkert *et al.*, 2008). EMSA analysis revealed that NarL directly regulates *rsmY* and *rsmZ* through specifically binding their promoter regions, though further work is required to determine how this influences GacA–IHF regulation of these small RNAs. GacA is a response regulator and the point of convergence for three sensor kinases, GacS, LadS and RetS, which interact to define the levels of free RsmA within the cell (Goodman *et al.*, 2004; Laskowski *et al.*, 2004; Ventre *et al.*, 2006; Zolfaghar *et al.*, 2005). The potential for cross-talk between the Gac–Anr–Rsm regulatory circuits may represent a molecular mechanism through which the cell can integrate temporal and infection-specific signals with exquisite precision to ensure an appropriately modulated adaptive response. It is interesting that both phosphorylated and unphosphorylated NarL bound to the *rsmY*, *rsmZ* and *narK* promoters. While the *E. coli* and *Salmonella*

Typhi NarL proteins have been shown to bind only when phosphorylated, no such data are available for *P. aeruginosa* NarL. Furthermore, *P. aeruginosa* NarL is only 56 % similar to both *E. coli* and *S. Typhi*, while they are 95 % similar to each other. Taken together with our EMSA analysis, this suggests that the requirement for phosphorylation may not be a feature of *P. aeruginosa* NarL–DNA binding, although it may be important for its mode of regulation. Future studies will seek to uncover the molecular mechanisms and specificity underlying the influence of NarL on *rsmY* and *rsmZ* expression.

We previously reported that RsmA positively regulates the T3SS in aerobic conditions, and here we show that the microaerobic induction of the T3SS is also dependent on RsmA. In light of this, we considered the possibility that oxygen availability may influence the expression of RsmA-regulated genes other than those of the T3SS. This was found to be the case for the *pilMNOPQ* operon, which was found to be induced under limiting oxygen conditions (J. O’Callaghan, unpublished data). However, comparative transcriptomic analysis revealed that three anaerobic-responsive genes, *PA1414*, *PA1673* and *oprG*, are negatively regulated by RsmA (Brencic & Lory, 2009) while being positively influenced by Anr (Trunk *et al.*, 2010), suggesting that other regulatory factors may influence the

Fig. 4. Model for modulation of the *P. aeruginosa* T3SS by low oxygen. Anr undergoes structural changes in response to limiting oxygen that cause it to directly activate its regulon, which includes the *narXL* two-component system. NarL subsequently inhibits *rsmYZ* transcription through direct binding of the respective promoter regions, allowing free RsmA to positively modulate the T3SS in response to limiting oxygen. RsmA is thought to directly modulate the T3SS through regulation of *exsA*. ExsA functions as a positive transcriptional regulator of the T3SS, and its activity is controlled by the ExsE–ExsC–ExsD anti-activator cascade. Further interplay between the Anr/NarL and RsmA/Y/Z regulatory networks is evident in Anr (positive) and RsmA (negative) modulation of targets such as the *hcnABC* promoter. Previous studies have demonstrated that GacS, LadS and RetS interact with the GacA global regulator, which also binds and modulates *rsmYZ* promoters, dictating free RsmA pools. This raises the intriguing question of whether these host-responsive two-component systems interface with the Anr/NarL pathway under microaerobic/anaerobic growth conditions. Arrowheads indicate positive regulation, blunt ends indicate negative regulation, solid lines indicate direct regulation, dashed lines indicate indirect regulation.

relationship between these distinct yet interfacing pathways. Furthermore, both Anr and RsmA have been shown to regulate HCN production in a mutually exclusive manner (Pessi & Haas, 2000; Pessi *et al.*, 2001). Anr positively regulates *hcnABC* promoter activity, while RsmA binds the transcript to repress its translation. Based on these studies one would expect there to be more overlap between the transcriptomes of the *anr* and *rsmA* mutants, which suggests that experimental conditions may be crucial when using transcriptome profiling to study RsmA regulation. However, the RsmA regulatory profiles do exhibit substantial overlap with the profiles from microaerobic growth conditions in biofilms and in artificial sputum media (Fung *et al.*, 2010; Waite & Curtis, 2009), whereby the transcriptional regulator PtxS, and genes associated with phenazine, PQS production and early biofilm formation, are modified in common. It has been reported that a subtle environmental difference can exert significant variation in the control of target genes of the Gac/Rsm pathway (Ventre *et al.*, 2006), and therefore a fine balance can exist between positive and negative regulation of target genes by RsmA. Given that RsmA is a regulator of secondary metabolites and is central to carbon metabolism, metabolic balance within the cell may be an additional factor that influences the interplay between the RsmA/Y/Z and Anr/NarL pathways, and this warrants further investigation.

The physiological relevance of these findings during infection remains to be elucidated, as typically strains isolated from CF patients infected long-term with *P. aeruginosa* are type III-defective due to mutation of regulatory genes during chronic/late infection (Mena *et al.*, 2008; Smith *et al.*, 2006), while stationary phase growth in artificial sputum media correlates with reduced T3SS production (Fung *et al.*, 2010). Therefore, while the T3SS-inducing conditions of limiting oxygen may exist throughout chronic infection, it is possible that activation of the T3SS under these conditions may be restricted to early chronic infection. However, it is intriguing to speculate that a subpopulation of cells may retain an intact T3SS, which would remain active in the limiting oxygen conditions.

In conclusion, this study presents the novel finding that the T3SS of *P. aeruginosa* is induced in response to limiting oxygen, a physiologically relevant condition in bacterial biofilm structures and CF lung mucus. The inducing regulatory mechanism is revealed as a hierarchical signal transduction cascade, whereby limiting oxygen triggers Anr and NarL, NarL represses *rsmY* and *rsmZ* promoter activity, and free RsmA accumulates to positively regulate the T3SS and potentially other RsmA-regulated virulence determinants. Further study is warranted to determine the precise NarL-binding site in the *rsmY* and *rsmZ* promoters, and the exact mechanism by which NarL binding down-regulates *rsmY* and *rsmZ*. The suggestion that Anr–NarL and RsmAYZ form part of a newly defined limiting oxygen-responsive pathway provides further evidence that

RsmA is a global regulator of virulence, and may act as a common point of convergence in the *P. aeruginosa* response to diverse environmental triggers. Furthermore, *P. aeruginosa* growth during chronic infection may be microaerobic or even anaerobic, leading to a shift in our understanding of the molecular mechanisms that underpin the acute and chronic stages of *P. aeruginosa* infection (Hoboth *et al.*, 2009). As a result, exogenous oxygen availability is emerging as an important environmental factor in *P. aeruginosa* pathogenesis, influencing key cellular activities such as metabolic adaptation and virulence factor production. While considerable attention has focused on the signal transduction mechanisms and phenotypes that influence the host–microbe interaction during infection, the experimental design has largely been biased towards aerobic growth. However, the emerging ‘microaerobic paradigm’ may necessitate a reappraisal of the established norms regarding virulence factor production, biofilm formation and antibiotic resistance in this and other important pathogens.

ACKNOWLEDGEMENTS

This research was supported in part by grants awarded by the European Commission (MTKD-CT-2006-042062; O36341; Marie Curie TOK : TRAMWAYS), the Science Foundation of Ireland (SFI 07/IN.I/B948; 08/RFP/GEN1295; 09/RFP/BMT2350), the Department of Agriculture and Food (DAF RSF 06 321; DAF RSF 06 377), the Irish Research Council for Science, Engineering and Technology (IRCSET) (05/EDIV/FP107/INTERPAM), the Health Research Board (RP/2004/145; RP/2006/271; RP/2007/290; RP; HRA/2009/146) the Environmental Protection Agency (EPA 2006-PhD-S-21) and the Higher Education Authority of Ireland (PRTL13; PRTL4). We thank Frederick M. Ausubel, Massachusetts General Hospital, Boston, MA, USA, for supplying strains, Barbara I. Kazmierczak, Yale University, New Haven, CT, USA, and Herbert P. Schweizer, Colorado State University, Fort Collins, CO, USA, for providing plasmids, and Ina Attree and Alan Hauser for contributing antibodies. We also thank Pat Higgins, Dr Olivia Rice and Dr Andrea Campisano for excellent technical assistance, and Dr Marlies Mooij for her intellectual input.

REFERENCES

- Alvarez-Ortega, C. & Harwood, C. S. (2007). Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. *Mol Microbiol* **65**, 153–165.
- Ando, H., Abe, H., Sugimoto, N. & Tobe, T. (2007). Maturation of functional type III secretion machinery by activation of anaerobic respiration in enterohaemorrhagic *Escherichia coli*. *Microbiology* **153**, 464–473.
- Becher, A. & Schweizer, H. P. (2000). Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal *lacZ* and *lux* gene fusions. *Biotechniques* **29**, 948–950, 952.
- Benkert, B., Quäck, N., Schreiber, K., Jaensch, L., Jahn, D. & Schobert, M. (2008). Nitrate-responsive NarX–NarL represses arginine-mediated induction of the *Pseudomonas aeruginosa* arginine fermentation *arcDABC* operon. *Microbiology* **154**, 3053–3060.
- Boucher, R. C. (2004). New concepts of the pathogenesis of cystic fibrosis lung disease. *Eur Respir J* **23**, 146–158.

- Brencic, A. & Lory, S. (2009). Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. *Mol Microbiol* **72**, 612–632.
- Cowell, B. A., Evans, D. J. & Fleiszig, S. M. (2005). Actin cytoskeleton disruption by ExoY and its effects on *Pseudomonas aeruginosa* invasion. *FEMS Microbiol Lett* **250**, 71–76.
- Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci U S A* **76**, 1648–1652.
- Fink, R. C., Evans, M. R., Porwollik, S., Vazquez-Torres, A., Jones-Carson, J., Troxell, B., Libby, S. J., McClelland, M. & Hassan, H. M. (2007). FNR is a global regulator of virulence and anaerobic metabolism in *Salmonella enterica* serovar Typhimurium (ATCC 14028s). *J Bacteriol* **189**, 2262–2273.
- Fung, C., Naughton, S., Turnbull, L., Tingpej, P., Rose, B., Arthur, J., Hu, H., Harmer, C., Harbour, C. & other authors (2010). Gene expression of *Pseudomonas aeruginosa* in a mucin-containing synthetic growth medium mimicking cystic fibrosis lung sputum. *J Med Microbiol* **59**, 1089–1100.
- Garrity-Ryan, L., Kazmierczak, B., Kowal, R., Comolli, J., Hauser, A. & Engel, J. N. (2000). The arginine finger domain of ExoT contributes to actin cytoskeleton disruption and inhibition of internalization of *Pseudomonas aeruginosa* by epithelial cells and macrophages. *Infect Immun* **68**, 7100–7113.
- Geiser, T. K., Kazmierczak, B. I., Garrity-Ryan, L. K., Matthay, M. A. & Engel, J. N. (2001). *Pseudomonas aeruginosa* ExoT inhibits *in vitro* lung epithelial wound repair. *Cell Microbiol* **3**, 223–236.
- Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S. & Lory, S. (2004). A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev Cell* **7**, 745–754.
- Hassett, D. J., Cuppoletti, J., Trapnell, B., Lyman, S. V., Rowe, J. J., Yoon, S. S., Hilliard, G. M., Parvatiyar, K., Kamani, M. C. & other authors (2002). Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. *Adv Drug Deliv Rev* **54**, 1425–1443.
- Hauser, A. R. (2009). The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat Rev Microbiol* **7**, 654–665.
- Hauser, A. R., Cobb, E., Bodi, M., Mariscal, D., Vallès, J., Engel, J. N. & Rello, J. (2002). Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by *Pseudomonas aeruginosa*. *Crit Care Med* **30**, 521–528.
- Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. & Schweizer, H. P. (1998). A broad-host-range FLP-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**, 77–86.
- Hoboth, C., Hoffmann, R., Eichner, A., Henke, C., Schmoldt, S., Imhof, A., Heesemann, J. & Hogardt, M. (2009). Dynamics of adaptive microevolution of hypermutable *Pseudomonas aeruginosa* during chronic pulmonary infection in patients with cystic fibrosis. *J Infect Dis* **200**, 118–130.
- Humair, B., Wackwitz, B. & Haas, D. (2010). GacA-controlled activation of promoters for small RNA genes in *Pseudomonas fluorescens*. *Appl Environ Microbiol* **76**, 1497–1506.
- Imhof, A. & Heinzer, I. (1996). Continuous monitoring of oxygen concentrations in several systems for cultivation of anaerobic bacteria. *J Clin Microbiol* **34**, 1646–1648.
- Jacobs, M. A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C. & other authors (2003). Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **100**, 14339–14344.
- Kay, E., Humair, B., Dénervaud, V., Riedel, K., Spahr, S., Eberl, L., Valverde, C. & Haas, D. (2006). Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *J Bacteriol* **188**, 6026–6033.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.
- Laskowski, M. A., Osborn, E. & Kazmierczak, B. I. (2004). A novel sensor kinase–response regulator hybrid regulates type III secretion and is required for virulence in *Pseudomonas aeruginosa*. *Mol Microbiol* **54**, 1090–1103.
- Liberati, N. T., Urbach, J. M., Miyata, S., Lee, D. G., Drenkard, E., Wu, G., Villanueva, J., Wei, T. & Ausubel, F. M. (2006). An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc Natl Acad Sci U S A* **103**, 2833–2838.
- Marteyn, B., West, N. P., Browning, D. F., Cole, J. A., Shaw, J. G., Palm, F., Mounier, J., Prévost, M. C., Sansonetti, P. & Tang, C. M. (2010). Modulation of *Shigella* virulence in response to available oxygen *in vivo*. *Nature* **465**, 355–358.
- Matsui, H., Wagner, V. E., Hill, D. B., Schwab, U. E., Rogers, T. D., Button, B., Taylor, R. M., II, Superfine, R., Rubinstein, M. & other authors (2006). A physical linkage between cystic fibrosis airway surface dehydration and *Pseudomonas aeruginosa* biofilms. *Proc Natl Acad Sci U S A* **103**, 18131–18136.
- Mena, A., Smith, E. E., Burns, J. L., Speert, D. P., Moskowitz, S. M., Perez, J. L. & Oliver, A. (2008). Genetic adaptation of *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients is catalyzed by hypermutation. *J Bacteriol* **190**, 7910–7917.
- Mooij, M. J., O'Connor, H. F., Tian, Z. X., Wang, Y. P., Adams, C., Morrissey, J. P. & O'Gara, F. (2010). Antibiotic selection leads to inadvertent selection of *nfxC*-type phenotypic mutants in *Pseudomonas aeruginosa*. *Environ Microbiol* **2**, 461–464.
- Mulcahy, H., O'Callaghan, J., O'Grady, E. P., Adams, C. & O'Gara, F. (2006). The posttranscriptional regulator RsmA plays a role in the interaction between *Pseudomonas aeruginosa* and human airway epithelial cells by positively regulating the type III secretion system. *Infect Immun* **74**, 3012–3015.
- Palmer, K. L., Brown, S. A. & Whiteley, M. (2007). Membrane-bound nitrate reductase is required for anaerobic growth in cystic fibrosis sputum. *J Bacteriol* **189**, 4449–4455.
- Pessi, G. & Haas, D. (2000). Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhIR in *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 6940–6949.
- Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M. T., Cámara, M., Haas, D. & Williams, P. (2001). The global posttranscriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 6676–6683.
- Sawers, R. G. (1991). Identification and molecular characterization of a transcriptional regulator from *Pseudomonas aeruginosa* PAO1 exhibiting structural and functional similarity to the FNR protein of *Escherichia coli*. *Mol Microbiol* **5**, 1469–1481.
- Schreiber, K., Krieger, R., Benkert, B., Eschbach, M., Arai, H., Schobert, M. & Jahn, D. (2007). The anaerobic regulatory network required for *Pseudomonas aeruginosa* nitrate respiration. *J Bacteriol* **189**, 4310–4314.

- Schüller, S. & Phillips, A. D. (2010). Microaerobic conditions enhance type III secretion and adherence of enterohaemorrhagic *Escherichia coli* to polarized human intestinal epithelial cells. *Environ Microbiol* **12**, 2426–2435.
- Sharma, V., Noriega, C. E. & Rowe, J. J. (2006). Involvement of NarK1 and NarK2 proteins in transport of nitrate and nitrite in the denitrifying bacterium *Pseudomonas aeruginosa* PAO1. *Appl Environ Microbiol* **72**, 695–701.
- Smith, E. E., Buckley, D. G., Wu, Z., Saenphimmachak, C., Hoffman, L. R., D'Argenio, D. A., Miller, S. I., Ramsey, B. W., Speert, D. P. & other authors (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci U S A* **103**, 8487–8492.
- Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E. & Lugtenberg, B. J. J. (1987). Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1J1. *Plant Mol Biol* **9**, 27–39.
- Stewart, P. S. & Franklin, M. J. (2008). Physiological heterogeneity in biofilms. *Nat Rev Microbiol* **6**, 199–210.
- Swinger, K. K. & Rice, P. A. (2004). IHF and HU: flexible architects of bent DNA. *Curr Opin Struct Biol* **14**, 28–35.
- Tian, Z. X., Mac Aogáin, M., O'Connor, H. F., Fargier, E., Mooij, M. J., Adams, C., Wang, Y. P. & O'Gara, F. (2009). MexT modulates virulence determinants in *Pseudomonas aeruginosa* independent of the MexEF-OprN efflux pump. *Microb Pathog* **47**, 237–241.
- Trunk, K., Benkert, B., Quäck, N., Münch, R., Scheer, M., Garbe, J., Jänsch, L., Trost, M., Wehland, J. & other authors (2010). Anaerobic adaptation in *Pseudomonas aeruginosa*: definition of the Anr and Dnr regulons. *Environ Microbiol* **12**, 1719–1733.
- Tyson, K. L., Bell, A. I., Cole, J. A. & Busby, S. J. (1993). Definition of nitrite and nitrate response elements at the anaerobically inducible *Escherichia coli* *nirB* promoter: interactions between FNR and NarL. *Mol Microbiol* **7**, 151–157.
- Ventre, I., Goodman, A. L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., Bleves, S., Lazdunski, A., Lory, S. & Filloux, A. (2006). Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A* **103**, 171–176.
- Waite, R. D. & Curtis, M. A. (2009). *Pseudomonas aeruginosa* PAO1 pyocin production affects population dynamics within mixed-culture biofilms. *J Bacteriol* **191**, 1349–1354.
- Wolfgang, M. C., Lee, V. T., Gilmore, M. E. & Lory, S. (2003). Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. *Dev Cell* **4**, 253–263.
- Worlitzsch, D., Tarran, R., Ulrich, M., Schwab, U., Cekici, A., Meyer, K. C., Birrer, P., Bellon, G., Berger, J. & other authors (2002). Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J Clin Invest* **109**, 317–325.
- Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A., Parvatiyar, K., Kamani, M. C., Allen, H. L., DeKievit, T. R., Gardner, P. R. & Schwab, U. (2002). *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev Cell* **3**, 593–603.
- Zhang, X. & Bremer, H. (1995). Control of the *Escherichia coli* *rrnB* P1 promoter strength by ppGpp. *J Biol Chem* **270**, 11181–11189.
- Zimmermann, A., Reimann, C., Galimand, M. & Haas, D. (1991). Anaerobic growth and cyanide synthesis of *Pseudomonas aeruginosa* depend on *anr*, a regulatory gene homologous with *fnr* of *Escherichia coli*. *Mol Microbiol* **5**, 1483–1490.
- Zolfaghar, I., Angus, A. A., Kang, P. J., To, A., Evans, D. J. & Fleiszig, S. M. (2005). Mutation of *retS*, encoding a putative hybrid two-component regulatory protein in *Pseudomonas aeruginosa*, attenuates multiple virulence mechanisms. *Microbes Infect* **7**, 1305–1316.

Edited by: W. Bitter