Microbiology (2012), **158**, 353–367 DOI 10.1099/mic.0.053371-0

Serum albumin alters the expression of iron-controlled genes in *Pseudomonas aeruginosa*

Cassandra Kruczek,[1] Mitchell Wachtel,[2] Magdy S. Alabady,[3] Paxton R. Payton,[4] Jane A. Colmer-Hamood[1] and Abdul N. Hamood[1]

Correspondence
Abdul N. Hamood
abdul.hamood@ttuhsc.edu

[1]Department of Microbiology & Immunology, School of Medicine, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA

[2]Department of Pathology, School of Medicine, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA

[3]Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

[4]United States Department of Agriculture, Cropping Systems Research Laboratory, The Plant Stress and Germplasm Development Unit, Lubbock, TX 79415, USA

Pseudomonas aeruginosa, which causes serious infections in immunocompromised patients, produces numerous virulence factors, including exotoxin A and the siderophore pyoverdine. As production of these virulence factors is influenced by the host environment, we examined the effect serum has on global transcription within *P. aeruginosa* strain PAO1 at different phases of growth in an iron-deficient medium. At early exponential phase, serum significantly enhanced expression of 138 genes, most of which are repressed by iron, including *pvdS*, *regA* and the pyoverdine synthesis genes. However, serum did not interfere with the repression of these genes by iron. Serum enhanced *regA* expression in a *fur* mutant of PAO1 but not in a *pvdS* mutant. The serum iron-binding protein apotransferrin, but not ferritin, enhanced *regA* and *pvdS* expression. However, in PAO1 grown in a chemically defined medium that contains no iron, serum but not apotransferrin enhanced *pvdS* and *regA* expression. While complement inactivation failed to eliminate this effect, albumin absorption reduced the effect of serum on *pvdS* and *regA* expression in the iron-deficient medium chelexed tryptic soy broth dialysate. Additionally, albumin absorption eliminated the effect of serum on *pvdS* and *regA* expression in the chemically defined medium. These results suggest that serum enhances the expression of *P. aeruginosa* iron-controlled genes by two mechanisms: one through apotransferrin and another one through albumin.

Received 15 July 2011
Revised 3 October 2011
Accepted 30 October 2011

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative pathogen that causes acute infections in immunocompromised hosts, including severely burned patients, cancer patients undergoing chemotherapy and HIV-infected patients (Branski *et al.*, 2009; Sadikot *et al.*, 2005). *P. aeruginosa* is also a major cause of chronic lung infections in patients with cystic fibrosis (Pier & Ramphal, 2005; Sadikot *et al.*, 2005). Damage caused by *P. aeruginosa* is due to the production of several cell-associated and extracellular virulence factors, such as the cell-associated flagellum, pili, lipopolysaccharide and alginate; and secreted exotoxin A (ETA), elastase, alkaline proteases and type III secretion factors (Pier & Ramphal, 2005; Sadikot *et al.*, 2005; van Delden, 2004). The production of these factors is controlled by the environment within different infection sites.

Iron, an element essential for the growth and metabolism of bacteria, is sequestered by iron-binding proteins, such as transferrin and lactoferrin, at infection sites within the host, (González-Chávez *et al.*, 2009; Wang & Pantopoulos, 2011). Consequently, during the infection process bacterial pathogens encounter iron-restricted conditions (Nairz *et al.*, 2010). In response, infecting bacteria produce different iron-acquisition systems (Anzaldi & Skaar, 2010; Cornelis, 2010; Nairz *et al.*, 2010). Iron-limited conditions trigger *P. aeruginosa* to produce several factors that are directly or indirectly involved in iron acquisition. These factors include the siderophores pyoverdine and pyochelin, proteases, ETA and haem utilization systems (Cornelis, 2010; Cornelis *et al.*, 2009; Hamood *et al.*, 2004). The production of these factors

Abbreviations: ABS, adult bovine serum; AHS, adult human serum; AHP, adult human plasma; ECF, extracytoplasmic function; ETA, exotoxin A; IS box, iron-starvation box; NE, norepinephrine; THA, therapeutic human albumin (10% Albuminar); TSB-DC, chelexed tryptic soy broth dialysate.

The GEO accession number for the microarray data associated with this paper is GSE30698.

Supplementary material is available with the online version of this paper.

by *P. aeruginosa* is a complicated process that involves numerous regulators (Hamood *et al.*, 2004). However, a key factor that regulates the activity of many of these different systems is the ferric uptake regulator (Fur) (Barton *et al.*, 1996; Cornelis *et al.*, 2009; Hamood *et al.*, 2004). Upon its activation by intracellular iron, Fur represses the expression of genes that code for iron-acquisition factors (Barton *et al.*, 1996; Cornelis *et al.*, 2009). Genes that are directly regulated by iron-activated Fur contain specific sequences within their upstream regions to which Fur specifically binds (Fur box) (Ochsner *et al.*, 1995, 1996; Ochsner & Vasil, 1996). Certain genes are regulated by Fur through a hierarchical system. For example, Fur represses the expression of *pvdS*, which codes for the extracytoplasmic function (ECF) sigma factor PvdS (Cornelis *et al.*, 2009; Ochsner *et al.*, 1996; Vasil & Ochsner, 1999). PvdS is, in turn, essential for the expression of the ETA gene (*toxA*) and the pyoverdine genes (Cornelis *et al.*, 2009; Hamood *et al.*, 2004; Ochsner & Vasil, 1996; Vasil & Ochsner, 1999). Similarly, Fur represses the expression of *pchR*, which is required for the expression of different genes involved in pyochelin production (Ochsner *et al.*, 1995). Besides Fur, *P. aeruginosa* may contain other proteins that play key roles in regulating the expression of different iron-acquisition systems. However, unlike Fur, which can be activated *in vitro* by low-iron conditions, some of these key regulation systems may be activated in response to specific environmental cues within the host.

One host factor likely to influence the production of *P. aeruginosa* virulence factors is serum. During systemic infections and within infected wounds, *P. aeruginosa* is exposed to serum or components of serum. We recently showed that human serum and plasma interfere with the development of *P. aeruginosa* biofilms on intravenous catheters (Hammond *et al.*, 2010). This hindrance appears to be related to the serum iron-binding protein transferrin (Ardehali *et al.*, 2003; Hammond *et al.*, 2010). Here we demonstrate that serum affects global gene expression in *P. aeruginosa* in the early exponential phase of growth, enhancing the expression of 154 genes, many of which are iron-controlled. The mechanism of enhancement is independent of iron and Fur, but requires functional PvdS. Serum fractionation and denaturation revealed that the enhancing activity lies within $\geqslant$50 kDa proteins. Depletion of albumin from the serum abrogated the enhancement while exogenous therapeutic human albumin solution reconstituted the effect. We propose that serum albumin plays a critical role in *P. aeruginosa* virulence during early phases of infection by enhancing the expression of iron-controlled genes through a Fur-independent mechanism that is not related to albumin-associated iron.

METHODS

Bacterial strains and plasmids, and growth media and conditions. Strains and plasmids utilized in this study are described in Table 1. For general growth experiments, strains were grown in the iron-containing medium Luria–Bertani (LB) broth (Miller, 1972). For all other analyses, *P. aeruginosa* strains were grown in either the iron-deficient medium TSB-DC (Chelex-treated trypticase soy broth dialysate) to which glycerol (1 % v/v) and monosodium glutamate (0.5 M) were added (Ohman *et al.*, 1980) or TSB-DC containing 10 % (v/v) adult bovine serum (TSB-DC/ABS), 10 % adult human serum (TSB-DC/AHS), adult human plasma (TSB-DC/AHP), or 10 %

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description*	Source and reference
Strains		
PAO1	Prototrophic PAO1	S. E. H. West, University of Wisconsin (West *et al.*, 1994)
C6	PAO1 parent strain; Δ*anr,* Fur mutation is Ala-10-Gly	M. L. Vasil, University of Colorado Denver (Barton *et al.*, 1996)
PAO6261	PAO1 parent strain; Δ*anr*	M. L. Vasil (Barton *et al.*, 1996)
PAO1 : : *pvdS*	PAO1 with 460 bp *pvdS* internal deletion, Gm^r	I. Lamont, University of Otago, New Zealand (Cunliffe *et al.*, 1995)
PW6979 : : *bfrB-lacZ*	*bfrB*-C04 : : IS*lacZ*/hah, Tc^r	University of Washington Genome Center (Jacobs *et al.*, 2003)
Plasmids		
pRL88	pSW205 carrying a *regA–lacZ* fusion, Cb^r	D. Storey, University of Calgary, Canada (Storey *et al.*, 1990)
pMP220 : : P*pvdA*	pMP220 carrying a *pvdA* promoter transcriptional *lacZ* fusion, Tc^r	P. Visca, University 'Roma Tre', Italy (Ambrosi *et al.*, 2002)
pMP220 : : P*pvdS*	pMP220 carrying a *pvdS* promoter transcriptional *lacZ* fusion, Tc^r	P. Visca (Ambrosi *et al.*, 2002)
pDH10	pMP190 carrying a *pchR–lacZ* fusion, Sm^r Cm^r	K. Poole, Queen's University, Canada (Heinrichs & Poole, 1996)

*Cb, carbenicillin; Cm, chloramphenicol; Gm, gentamicin; Sm, streptomycin; Tc, tetracycline; ^{r}, resistant.

Albuminar (CSL Behring) (TSB-DC/THA). Iron-sufficient medium was prepared by the addition of $FeCl_3$ to TSB-DC to a final concentration of 20 μg Fe^{3+} ml^{-1} (TSB-DC/Fe) (Ohman *et al.*, 1980). Norepinephrine (NE) was added to a concentration of 500 μM. Modified M9 medium (MM9) was prepared by the addition of glycerol (1 %, v/v) and monosodium glutamate (0.5 M) to the chemically defined medium M9 (Miller, 1972). Ferritin (Sigma-Aldrich) apotransferrin (R&D Systems) and holotransferrin (Sigma-Aldrich) were added to MM9 at concentrations ranging from 20 to 200 μg ml^{-1}. Antibiotics were added to the growth medium as needed: 200 μg carbenicillin ml^{-1}, 300 μg streptomycin ml^{-1} or 80 μg tetracycline ml^{-1} to maintain plasmid stability.

For transcriptome analysis and analysis of ETA and pyoverdine production, as well as the expression of different genes, *P. aeruginosa* strains were first grown overnight in LB broth at 37 °C. An aliquot of the overnight culture was pelleted, washed and resuspended in TSB-DC. The resuspended culture was used to inoculate fresh TSB-DC with or without serum, iron or other additives to an OD_{600} of 0.02–0.03 (4 mm cuvette pathlength; GENESYS 20, ThermoFisher Scientific). The cultures were grown at 37 °C to the indicated times with shaking at 230 r.p.m. Each experiment was conducted in triplicate (three separate flasks) with three replicates per flask.

Transcriptome analysis in the presence of serum. Triplicate cultures of PAO1 were grown at 37 °C with shaking in either TSB-DC or TSB-DC/ABS to an OD_{600} of 1.11 and 0.69 (early exponential phase of growth, 4 h time point) and 4.7 and 2.5 (early stationary phase, 8 h time point), respectively. As growth levels in TSB-DC/ABS were reduced compared to growth in TSB-DC, the TSB-DC cultures were adjusted to the same OD_{600} as their companion cultures in TSB-DC/ABS by dilution with fresh TSB-DC prior to RNA extraction. Extraction of RNA, and cDNA synthesis, fragmentation and labelling were conducted essentially as previously described (DeRisi, 2001; Garber & Urisman, 2001; Yang *et al.*, 2002). Labelled samples were hybridized to PAO1 genomic arrays provided by the Institute for Genomic Research (Rockville, MD; now part of the J. Craig Venter Institute) as part of the Pathogen Functional Genomics Center initiative. The hybridization, washing and drying were done using Corning hybridization chambers (Corning Life Sciences). Arrays were scanned using a GenePix 4200A array scanner (Molecular Devices). Genes were considered differentially regulated if the relative change was twofold or more between PAO1 grown in TSB-DC and TSB-DC/ABS at 4 or 8 h. Microarray data and metadata described in this study have been deposited in the GEO database (accession number GSE30698).

Assays for gene expression by β-galactosidase, ETA and pyoverdine. Assays for β-galactosidase were performed as previously reported (Gaines *et al.*, 2005; Miller, 1972; Stachel *et al.*, 1985). The calculation of relative units of β-galactosidase activity takes into account the level of growth. ETA levels within the supernatant of PAO1 were determined by sandwich ELISA as previously described (Gaines *et al.*, 2005). Values were standardized by dividing the amount of ETA in pg ml^{-1} by the OD_{600} of the culture from which the fraction was obtained. Pyoverdine levels within the supernatant fractions of PAO1 were quantified spectrophotometrically as A_{405} according to Stintzi *et al.* (1996). Normalization of pyoverdine levels for growth was obtained by dividing A_{405} by OD_{600}.

Collection and preparation of adult human serum and human plasma. Fifty millilitres of whole blood was obtained by venipuncture from each of five healthy volunteers under a protocol approved by the Institutional Review Board at Texas Tech University Health Sciences Center. The blood was drawn into three 10 ml glass tubes without additives for collection of serum and two 10 ml glass tubes containing 143 USP units of freeze-dried sodium heparin for collection of plasma (Becton Dickinson). For the separation of either serum or plasma from the cellular portion of the blood, the tubes were incubated at room temperature for 2 h. To achieve cell-free plasma (AHP) or serum (AHS), the tubes were then centrifuged at 3000 r.p.m. for 15 min at room temperature. Sera from the five donors were pooled, aliquoted and stored at −20 °C until use; the plasma samples were handled in the same manner.

Fractionation and treatment of serum. Using molecular mass cut-off membrane filters (Vivaspin) we separated ABS and AHS into different fractions ($\geqslant 3/<3$ kDa and $\geqslant 50/<50$ kDa). Albumin was absorbed from ABS or AHS using DEAE Affi-Gel Blue gel columns (Bio-Rad) according to the manufacturer's recommendations. To determine effects of protein degradation, serum was heated to 70 °C for 3 or 5 h and cooled to room temperature; or treated with 3 mg trypsin ml^{-1} (Sigma-Aldrich) for 1 h 37 °C and the reaction was terminated by addition of 3 mg trypsin inhibitor ml^{-1} (Sigma-Aldrich). Trypsin treatment was confirmed by comparing the protein profile of treated and non-treated samples by SDS-PAGE analysis (data not shown). Treated serum was utilized in the expression experiments as described above.

Immunoblotting experiments. These experiments were conducted as previously described (Schaber *et al.*, 2004) using a commercially available rabbit anti-human transferrin antibody (Rockland Immunochemicals) that detects both apo- and holotransferrin. Albumin absorbed from AHS was eluted from the DEAE Affi-Gel Blue gel column according to the manufacturer's instructions. Purified human apotransferrin and human holotransferrin were used as controls.

RESULTS

Serum alters the expression of numerous *P. aeruginosa* PAO1 genes during early exponential growth

To examine the effect of serum on *P. aeruginosa* gene expression, we selected the *P. aeruginosa* prototrophic strain PAO1, which has been extensively utilized in numerous *in vitro* and *in vivo* virulence studies (Peluso *et al.*, 2010; Schaber *et al.*, 2004; Smith & Iglewski, 2003). To establish the time points at which serum would affect gene expression, PAO1 was grown in the iron-limited medium TSB-DC with or without 10 % (v/v) ABS (TSB-DC/ABS) for 10 h. Samples were obtained every 2 h and the growth index was determined. The presence of 10 % ABS significantly reduced the growth of PAO1 at time points tested (Fig. 1). Despite this reduction, PAO1 grown in ABS reached early exponential phase at 4 h and early stationary phase at 8 h in both media (Fig. 1). Accordingly, we determined the PAO1 transcriptome in both media at these two specific time points. However, to standardize the comparison, we adjusted the cultures to the same OD_{600} (at each time point) by adding fresh TSB-DC to the TSB-DC cultures. Total RNA was extracted and reverse transcribed, and the transcriptome analysis was conducted as described in Methods. We identified 178 PAO1 genes whose expression was significantly altered (at least a twofold change) by the presence of serum only at the early exponential phase (4 h) of growth (see Supplementary Table S1, available with the online version of this paper). Of these genes, the expression of 138 was significantly enhanced while that of 40 genes was

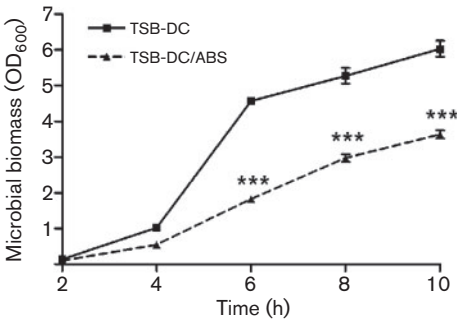

Fig. 1. Effect of ABS on *P. aeruginosa* growth. One-millilitre aliquots of PAO1 overnight culture in LB broth were pelleted, washed twice with TSB-DC, resuspended in TSB-DC and inoculated into fresh TSB-DC or TSB-DC containing 10% ABS (TSB-DC/ABS) to a starting OD_{600} of 0.02–0.03. Cultures were incubated at 37 °C with shaking at 230 r.p.m. Samples were obtained at 2 h intervals and the OD_{600} determined. Values represent the mean ± SEM of three independent experiments. Statistical significance was determined by one-way ANOVA with Tukey's post test. ***, $P<0.001$.

significantly reduced (Table S1). In contrast, the expression of only a few genes was significantly altered (reduced) at the early stationary (8 h) phase of growth (Table S1).

Results of the transcriptome analysis revealed that many of the genes whose expression is significantly altered at 4 h are iron-regulated genes (Table 2). However, the effect of ABS on the expression of these genes was opposite to that of iron, i.e. ABS increased the expression of iron-repressed genes and reduced the expression of iron-induced genes (Table 2). The increase in the expression of iron-repressed genes by ABS varied between eightfold (*pchE*) and 92-fold (*pvdF*) (Table 2). Several of these genes have been previously characterized while others code for hypothetical proteins (Table 2). Additionally, many of the genes are clustered in operons, including the *pvd*, *phu*, *pch* and *nar* operons (Fig. 2). While ABS enhanced the expression of every gene within certain operons, it enhanced the expression of only one or two genes within other operons (Fig. 2). ABS significantly repressed the expression of one iron-induced gene (the bacterioferritin gene, *bfrB*) (Table 2). Previously characterized *P. aeruginosa* iron-regulated genes contain specific sequences within their upstream regions to which the iron-regulatory protein Fur or the ECF sigma factor PvdS bind: the Fur box or the iron-starvation box (IS box), respectively (Cornelis *et al.*, 2009; Hamood *et al.*, 2004; Ochsner & Vasil, 1996; Vasil & Ochsner, 1999). A computer search of the upstream regions of the ABS-regulated, iron-controlled genes identified the presence of the Fur box or the IS box within the upstream regions of several of these genes (Table 2, Fig. 2).

Serum enhances the expression of iron-controlled *P. aeruginosa* genes

To validate the results of the transcriptome analysis, we utilized plasmids carrying *lacZ* transcriptional or translational fusions or PAO1 strains containing chromosomal transcriptional fusions with the iron-repressed genes *regA*, *pvdA*, *pvdS* and *pchR*, and the iron-induced gene *bfrB* (Table 2) (Hamood *et al.*, 2004; Ma *et al.*, 1999; Vasil & Ochsner, 1999; Wilderman *et al.*, 2004). Since the significant effect of ABS on the expression of these genes was detected in the early exponential phase of growth (Fig. 1), we determined the expression of these genes over 2–6 h of growth. To compensate for the growth-related bias (Fig. 1), the growth index (OD_{600}) was incorporated into the formula for calculating the units of β-galactosidase activity (Gaines *et al.*, 2005; Stachel *et al.*, 1985). Compared with PAO1, the presence of ABS significantly enhanced the expression of *pvdA* and *regA* at the indicated time points (Fig. 3a, b). In conjunction with its effect on expression of the pyoverdine genes, ABS significantly enhanced pyoverdine production by PAO1 in the mid-exponential and early stationary phases of growth (Fig. 4a), a reflection of the time required for translating and processing the pyoverdine synthesis proteins. Similarly, ETA production was also enhanced (Fig. 4b). As expression of *toxA*, *regA* and pyoverdine requires functional PvdS (Cornelis, 2010; Hamood *et al.*, 2004; Vasil & Ochsner, 1999), we confirmed that ABS significantly enhanced the expression of *pvdS* (Fig. 3c). Furthermore, we showed that ABS enhances the expression of the pyochelin regulatory gene *pchR* (Fig. 3d), which is regulated by Fur. In contrast, ABS significantly represses the expression of the iron-inducible *bfrB* gene at 4 and 6 h (Fig. 3e). As expected, addition of iron increased *bfrB* expression significantly at these time points (Fig. 3e).

Serum enhancement of target genes does not occur through iron

One possible explanation for the above results is that a serum factor interferes with the regulation of PAO1 genes by iron. As a result, iron-induced genes would be repressed and vice versa. In our experiments, cells within the initial inocula of PAO1 in TSB-DC or TSB-DC/ABS contained a residual pool of iron. Prior to their inoculation into the iron-deficient TSB-DC, PAO1 cells were grown overnight in the iron-containing medium LB broth. The intracellular pool of iron in these cells would be partially depleted at the 4 h time point and completely depleted at the 8 h time point. Therefore, in the early exponential phase of growth, ABS would increase the expression of these genes by interfering with the repressive effect of the residual pool of iron. However, by the early stationary phase of growth, the intracellular pool of iron is depleted and the target genes would no longer be regulated by either iron or the serum factor.

To examine this possibility, we tried two approaches. Firstly, we depleted the intracellular pool of iron in PAO1 by subculturing the cells twice in TSB-DC prior to examining the effect of ABS on gene expression. If the above scenario is correct, we would no longer detect the effect of ABS on gene expression in the early exponential

phase of growth. Alternatively, if the effect of ABS is iron independent, the genes would be regulated. Since *pvdS* regulates the expression of a number of iron-regulated genes, we examined *pvdS* expression as representative of the ABS-upregulated genes. The addition of 10 % ABS to the second TSB-DC subculture of PAO1/pPMP220::P*pvdS* still significantly enhanced *regA* expression in the early exponential phase of growth (Fig. 5a). Secondly, we determined whether exogenously added iron would override the serum enhancement in *pvdS* expression in the early exponential phase of growth (two independent regulatory mechanisms). We grew PAO1/pPMP220::P*pvdS* overnight in LB broth and subcultured it into TSB-DC, TSB-DC/ABS, TSB-DC/Fe and TSB-DC/ABS/Fe. Preliminary experiments showed that iron completely represses *pvdS* expression at this concentration (20 μg Fe ml^{-1}; data not shown). Control experiments showed that, when added separately, iron repressed *pvdS* expression (Fig. 5b). However, ABS did not enhance *pvdS* expression in TSB-DC containing iron (Fig. 5c). We obtained similar results when we used the two approaches to examine *pvdA* and *regA* expression (data not shown). Next, we examined the effect of serum on *pvdS* expression in a growth medium in which the level of iron is not sufficient to completely repress the expression of different genes (LB broth). The addition of ABS significantly enhanced *pvdS* expression in LB broth at 4 and 6 h (Fig. 5d). These results support the possibility that ABS regulates the expression of its target genes by an iron-independent mechanism.

We previously showed that the addition of norepinephrine (NE) to cultures of PAO1 in ABS significantly reduced the expression of the iron-regulated genes *pvdS*, *regA* and *toxA* (Li *et al.*, 2009). Based on this and other experiments, we suggested that NE functions as a siderophore by removing iron from an iron-binding protein in the serum, such as transferrin, and transferring it to PAO1 (Li *et al.*, 2009). As a result, the intracellular pool of iron increases, which in turn represses the expression of *pvdS*, *regA* and *toxA*. To further confirm that the observed influence of ABS on these genes is not directly related to iron, we examined the effect of exogenously added NE (500 μM) on *pvdS* expression in PAO1 in the early exponential phase of growth. While ABS enhanced *pvdS* expression as previously observed, this enhancement was eliminated at 2 h upon the addition of NE (Supplementary Fig. S1).

Seven of the serum-regulated genes contain a Fur box within their upstream regions (Table 2, Fig. 2), raising the possibility that Fur plays a role in the regulation of some of the serum-targeted genes. However, the above results also suggest that serum affects the expression of these genes independently of the Fur cofactor, iron (Fig. 5). Therefore, to explore a possible link between serum and Fur in regulating PAO1 genes, we utilized the PAO1 Fur-deficient mutant C6 (Barton *et al.*, 1996). C6, which was derived from the PAO1 *anr* mutant strain PAO6261 (Ye *et al.*, 1995), carries mutations in both the *anr* and *fur-6* genes (Table 1) (Barton *et al.*, 1996). Barton *et al.* (1996) previously demonstrated that C6 is the only relatively stable PAO1-*fur* mutant; with the exception of C6, all other Fur-deficient mutants generated directly from PAO1 reverted to the wild-type phenotype. Within C6, ETA production is deregulated with respect to iron (Barton *et al.*, 1996). Thus, a possible role for Fur in our observed results would be indicated if ABS failed to regulate its target genes in C6. We determined the level of *regA* expression in PAO6261 and C6 containing pRL88 grown in either TSB-DC or TSB-DC/ABS. At 4 and 5 h, ABS significantly enhanced *regA* expression in both strains, suggesting that the absence of a functional Fur may not interfere with the regulation of PAO1 genes by serum (Fig. 6). However, since the C6 *fur* mutant is only relatively stable, a definitive conclusion regarding the role of Fur in this phenomenon cannot be made at this time.

Based on the above results, serum may regulate the expression of *toxA*, *regA* and the pyoverdine genes individually by direct regulation. Alternatively, serum may regulate these genes indirectly by increasing the expression of their main regulator, *pvdS*. To examine this possibility, we determined whether ABS increases *regA* expression in PAO1Δ*pvdS*, which carries an internal deletion in *pvdS*. As expected, the lack of functional PvdS reduced *regA* expression significantly; however, comparable levels of *regA* expression were seen in TSB-DC and TSB-DC/ABS (data not shown). Similar results were obtained when we examined *toxA* and *pvdD* expression in PAO1Δ*pvdS* (data not shown), suggesting that serum regulates *regA* and the pyoverdine genes through *pvdS*.

Bovine serum albumin enhances the expression of PAO1 iron-repressed genes

The above results suggest that serum influences the expression of PAO1 genes through a unique mechanism that is PvdS-dependent but iron- and Fur-independent. To further understand this mechanism, we attempted to identify the potential serum factor(s) that affect(s) the expression of these genes. We first addressed the possibility that the serum factor is a small molecule that enters the PAO1 cytoplasm (passively or actively) and programmes the expression of different genes. We membrane-fractionated ABS using a 3 kDa MWCO membrane (Vivaspin). The fraction that passed through the membrane contains small molecules and possibly peptides. Strain PAO1/pPMP220::P*pvdS* was grown using the filtrate and retentate in place of serum. At both the 2 h and 4 h time points, the serum-induced enhancement in *pvdS* expression was eliminated from the filtrate ($<$3 kDa) but preserved within the retenate ($\geqslant$3 kDa) (data not shown). We then repeated the process using a 50 kDa MWCO membrane. Again, enhancement of *pvdS* expression was detected with the retentate but not the filtrate at both time points (Supplementary Fig. S2). To confirm that the potential factor is a protein, we denatured the serum proteins by either heat or trypsin treatment. Heat treatment eliminated the effect of serum on *regA* expression (1400 units of β-galactosidase activity were produced by

Table 2. PAO1 iron-controlled genes influenced by serum in the early exponential phase of growth

ORF*	Gene*	Function/class*	Fold change†	Fur box (FB) GATAATGATAATCATTATC‡ Iron starvation box (ISB) TAAAT-N_{16}-CGT§
PA0707	*toxR/regA*	Transcriptional regulator ToxR/RegA	14	
PA1317	*cyoA*	Cytochrome *o* ubiquinol oxidase subunit II	11	
PA2384	*nn*‖	Probable DNA-binding protein	27	
Pyoverdine synthesis and transport				
PA2385	*pvdQ*	PvdQ	18	
PA2386	*pvdA*	L-Ornithine N^5-oxygenase	90	ISB
PA2389	*pvdR*	Protein of unknown function	27	ISB
PA2390	*pvdT*	ABC transporter	20	
PA2391	*opmQ*	Probable outer-membrane protein	20	
PA2392	*pvdP*	PvdP	70	ISB
PA2393	*nn*	Probable dipeptidase precursor	59	ISB
PA2394	*pvdN*	PvdN	89	
PA2395	*pvdO*	PvdO	24	
PA2396	*pvdF*	Pyoverdine synthetase F	92	ISB
PA2397	*pvdE*	Pyoverdine biosynthesis protein PvdE	26	ISB
PA2398	*fpvA*	Ferripyoverdine receptor	28	
PA2399	*pvdD*	Pyoverdine synthetase D	30	
PA2413	*pvdH*	Diaminobutyrate-2-oxoglutarate aminotransferase	17	ISB
PA2425	*pvdG*	PvdG	51	
PA2426	*pvdS*	Sigma factor PvdS	37	FB (GtaAtTGAcAATCATTATC)
PA3531	***bfrB***	**Bacterioferritin**	**0.09**	**Fur-like binding sequence**
PA4159	*fepB*	Ferrienterobactin-binding periplasmic protein precursor	9	
Pyochelin synthesis and transport				
PA4168	*fpvB*	Second ferric pyoverdine receptor FpvB	10	
PA4218	*fptX*	Putative permease, AmpG paralogue	5.3	
PA4219	*fptC*	Hypothetical protein	5.8	
PA4220	*fptB*	Hypothetical protein	6.2	
PA4221	*fptA*	Fe(III)-pyochelin outer-membrane receptor precursor	10	FB (cATAATGATAAgCATTATC)
PA4222	*pchI*	Probable ATP-binding component of ABC transporter	5.65	
PA4223	*pchH*	Probable ATP-binding component of ABC transporter	3.12	
PA4225	*pchF*	Pyochelin synthetase	2.5	
PA4226	*pchE*	Dihydroaeruginoic acid synthetase	8	
PA4227	*pchR*	Transcriptional regulator PchR	13	FB (GgaAATGAgAtTtATTATC)
PA4228	*pchD*	Pyochelin biosynthesis protein D	22	
PA4229	*pchC*	Pyochelin biosynthesis protein C	18	
PA4230	*pchB*	Salicylate biosynthesis protein PchB	18	
PA4231	*pchA*	Salicylate biosynthesis isochorismate synthase	9	
PA4359	*feoA*	Ferrous iron transport protein A	11.5	
PA4468	*sodM*	Superoxide dismutase	13	FB (GATAATGAgAtTgATTATt)
PA4469	*unc*¶	Hypothetical protein	46	
PA4470	*fumC1*	Fumarate hydratase	68	
PA4471	*unc*	Hypothetical protein	90	
Haem transport and utilization				
PA4705	*phuW*	Hypothetical protein	4.6	
PA4706	*phuV*	Haemin importer ATP binding subunit	7.9	
PA4707	*phuU*	Permease of ABC transporter	12.5	
PA4708	*phuT*	Haem-transport protein	21	FB (GcTAATGcaAATaATTATC)

Table 2. cont.

ORF*	Gene*	Function/class*	Fold change†	Fur box (FB) GATAATGATAATCATTATC‡ Iron starvation box (ISB) TAAAT-N_{16}-CGT§
PA4709	*phuS*	Haemin degrading factor	20	
PA4710	*phuR*	Haem/haemoglobin uptake outer membrane receptor precursor	32	FB (GATAATtATttgCATTAgC)
PA5531	*tonB1*	TonB1 protein	6	FB (ctgAATGATAATaATTATC)

*Information obtained from the *Pseudomonas* Genome Database$_{V2}$ (http://www.pseudomonas.com/) (Winsor *et al.*, 2009).
†PAO1 grown in TSB-DC/ABS compared to PAO1 grown in TSB-DC.
‡Consensus sequence for Fur box (Ochsner *et al.*, 2002).
§Consensus sequence for iron starvation box (Wilson *et al.*, 2001).
||Not named.
¶Unclassified.

PAO1/pRL88 in untreated serum and 14 units in heat-treated serum, a 101-fold reduction in activity). We obtained similar results with trypsin treatment (units of β-galactosidase activity produced by PAO1/pRL88 were reduced eightfold in trypsin-treated serum). We eliminated the possibility that the complement system is involved in the observed effect of serum by heating the serum at 65 °C for 20 min to inactivate complement. Complement-inactivated serum still increased *regA* expression in PAO/pRL88 at the 2 and 4 h time points (data not shown).

Albumin, a protein of approximately 65 kDa in its monomeric form, is a major component of serum (Foster, 1977; Peters, 1996; Rondeau & Bourdon, 2011). Previous studies suggested that serum albumin affects PAO1 virulence (Foster, 1977; Peters, 1996; Rondeau & Bourdon, 2011). Thus, to determine if serum albumin is the serum factor that affects the expression of PAO1 genes, we depleted the albumin from ABS using DEAE Affi-Gel Blue gel affinity columns. Compared with the effect of intact serum, albumin depletion eliminated the ABS-induced enhancement in *pvdS* expression in PAO1/pPMP220::P*pvdS* at both 2 h and 4 h (Fig. 7a), suggesting that serum albumin plays a major role in ABS enhancement of PAO1 gene expression.

Human serum and serum albumin produce the same effect on *regA* and *pvdS* expression

We then determined whether similar results would be obtained using adult human serum (AHS) and adult human plasma (AHP). Blood samples were collected from healthy volunteers; the serum or plasma fractions were separated and pooled, and tested as described in the above sections. Both AHS and AHP significantly enhanced *pvdS* and *regA* expression at both the 2 h and 4 h time points (Supplementary Fig. S3a, b). We then depleted the albumin from AHS using Affi-Gel Blue gel affinity columns. Compared with AHS, albumin depletion abrogated the effect on *pvdS* (Fig. 7b) and *regA* expression (data not shown) at both 2 h and 4 h. To further confirm the role of serum albumin, we examined the effect of the therapeutic human albumin (THA) solution Albuminar, which contains 10 % human albumin in normal saline. The THA enhanced expression of both *pvdS* and *regA* (Fig. 8a, b). These results strongly suggest that at early stages of growth of PAO1, human serum albumin is the serum factor that significantly increases the expression of several of *P. aeruginosa* iron-repressed genes.

Human albumin and apotransferrin each enhance the expression of PAO1 iron-controlled genes

Serum contains many iron-binding proteins, including transferrin, ferritin and albumin. Although TSB-DC is iron-deficient (Ohman *et al.*, 1980), our recent analysis revealed that the medium contains a trace amount of iron (0.21 μg ml^{-1}). Serum iron-binding proteins may chelate this trace amount of iron and enhance *pvdS* and *regA* expression. Therefore, we examined the effect of purified human ferritin, holotransferrin and apotransferrin on PvdS expression. At 20 μg ml^{-1}, none of the proteins enhanced *pvdS* expression (data not shown), while at 80 μg ml^{-1} and higher, only apotransferrin enhanced *pvdS* expression, by about four-fold (322 units of β-galactosidase activity for PAO1/pPMP220::P*pvdS* grown in TSB-DC vs 1307 units for PAO1/pPMP220::P*pvdS* grown in TSB-DC/apoTF). To determine if serum enhances *pvdS* expression independently of apotransferrin, we grew PAO1/pPMP220::P*pvdS* in MM9, which contains no iron. Even at 200 μg ml^{-1}, apotransferrin did not enhance *pvdS* expression (Fig. 9). However, serum significantly enhanced *pvdS* expression (Fig. 9). Serum also significantly enhanced *regA* expression PAO1/pRL88 grown in MM9 (data not shown). More importantly, albumin-deficient human serum failed to enhance *pvdS* expression in MM9 (Fig. 9), indicating that the effect of albumin is not directly related to iron. To exclude the possibility that passage of serum through the DEAE Affi-Gel Blue gel column eliminated both albumin and apotransferrin,

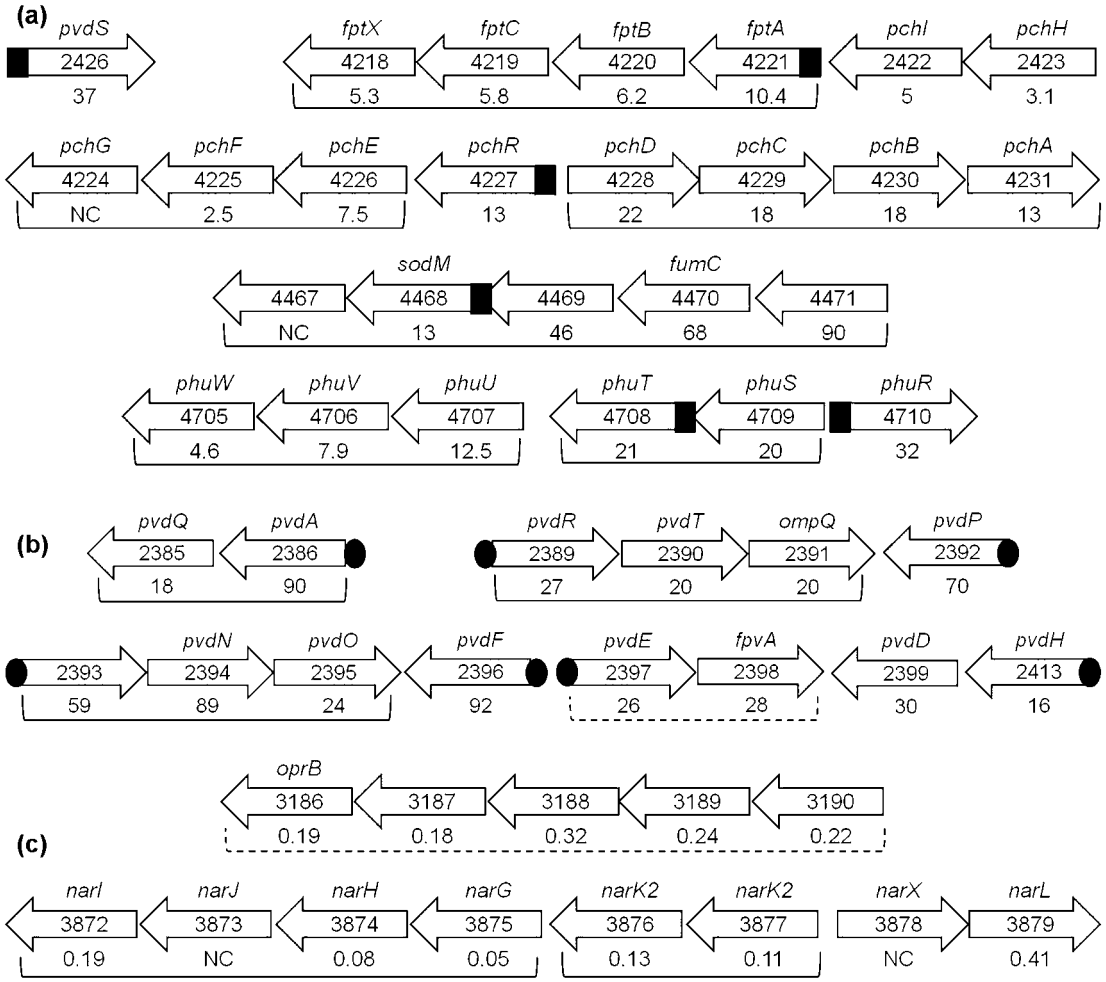

Fig. 2. Diagram of PAO1 iron-controlled genes enhanced or repressed by serum. The arrows with gene number indicate the orientation of the gene on the PAO1 chromosome (Pseudomonas Genome Database$_{V2}$; http://www.Pseudomonas.com; Winsor *et al.*, 2009). Gene names, if known, are above the arrow, and the fold change is below the arrow. Brackets indicate that genes lie in operons; dashed brackets indicate putative operons. (a) Genes/operons enhanced by serum and directly or indirectly regulated by Fur; black square indicates Fur box (Table 2). (b) Genes/operons enhanced by serum and directly or indirectly regulated by PvdS; black oval indicates iron starvation box (Table 2). (c) Genes/operons repressed by serum. The upstream region of *bfrB* contains a Fur box-like sequence.

we eluted the absorbed albumin-rich fraction and examined it for the presence of apotransferrin by immunoblotting. The albumin-rich fraction contained neither apo- nor holotransferrin (Fig. 10). Using the same approach, we also showed Albuminar contains neither form of transferrin (Fig. 10). These results suggest that the observed enhancement in *pvdS*/*regA* expression, and possibly the expression of other iron-controlled genes, by serum occurs through two separate mechanisms: one is apotransferrin-dependent (iron chelation) while the other is albumin-dependent.

DISCUSSION

Results presented in this study suggest a novel mechanism through which serum regulates the expression of different *P. aeruginosa* genes. The essential feature of this mechanism is its occurrence at early stages of growth and under iron-limited conditions. As shown by the results of the microarray analysis and confirmed by the transcriptional and translational fusion analyses, many of the serum-regulated genes are involved in either iron acquisition, iron transport or iron storage (Table 2, Fig. 2). However, although several of these serum-regulated genes also contain a Fur box within their upstream regions, their regulation by serum is opposite to their regulation by iron (Table 2). Several of these serum-regulated genes also contain a Fur box within their upstream regions (Table 2). Many genes that do not contain the Fur box contain an IS box and are regulated by PvdS, which carries a Fur box. Despite this, their regulation by serum does not appear to be directly related to iron. Our results showed that the phenomenon did not occur in response to the

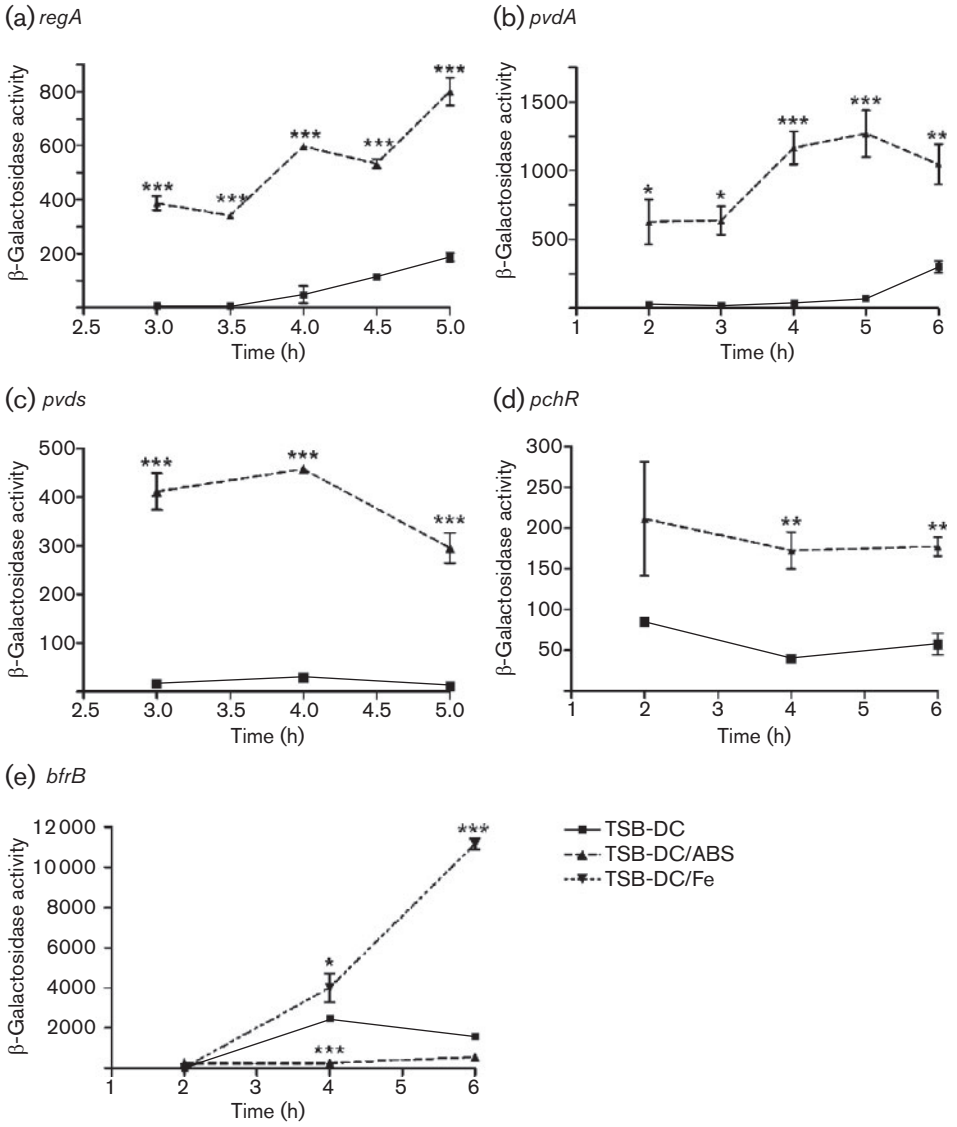

Fig. 3. Serum enhances the expression of *regA*, *pvdA*, *pvdS* and *pchR* but represses the expression of *bfrB* in the early exponential phase of growth. PAO1 containing transcriptional or translational *lacZ* fusion plasmids was grown in TSB-DC or TSB-DC/ABS as described in Fig. 1. Samples were collected at the time points indicated on the graphs and the *β*-galactosidase activity was determined and normalized for growth (see Methods). Plasmids pRL88, pMP220::P*pvdA*, pMP220::P*pvdS* and pDH10 were utilized to examine the expression of *regA* (a), *pvdA* (b), *pvdS* (c) and *pchR* (d), respectively. Strain PW6979::*bfrB-lacZ* was used to examine *bfrB* expression (e). Iron was added to TSB-DC at 5 μg ml^{-1} (TSB-DC/Fe) to induce *bfrB* expression as a control. Values represent the means ± SEM of three independent experiments. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$.

depletion of the intracellular pool of iron (Fig. 5a). Similarly, serum did not interfere with the repression of these genes by exogenous iron (Fig. 5c). Additionally, serum did not interfere with the repression of these two genes by NE (Fig. S1). Part of the observed effect is produced by the serum iron-binding protein apotransferrin, which chelated the trace amount of iron present in TSB-DC. However, serum significantly induced *pvdS* and *regA* expression even when PAO1 was grown in the chemically defined medium MM9, which contains no iron (Fig. 9). Therefore, it is unlikely that the observed serum-induced regulation of these genes is due only to the iron-chelating activity of apotransferritin within the serum. With respect to Fur, our results using the C6 *fur* mutant showed that the loss of functional Fur did not interfere with the regulation of PAO1 *pvdS* or *regA* by serum (Fig. 6). However, this conclusion must be tempered by the relative instability of this mutant.

Our experimental evidence strongly suggests that serum albumin regulates the expression of different *P. aeruginosa*

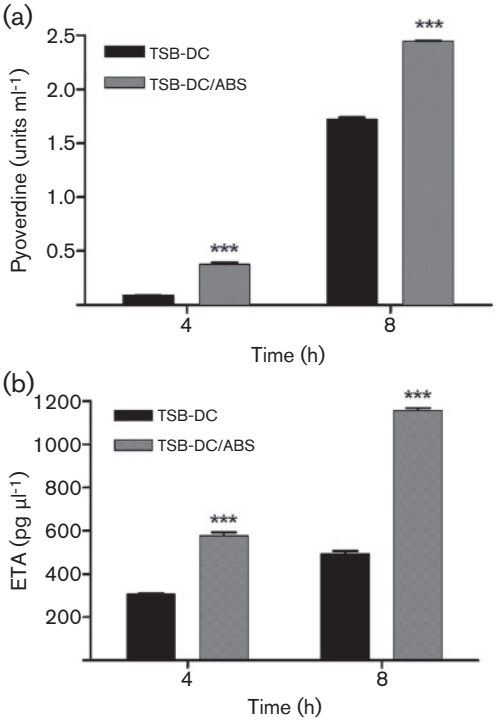

Fig. 4. Serum enhances synthesis of pyoverdine and exotoxin A (ETA). (a) To assess pyoverdine production, PAO1 was grown in TSB-DC and TSB-DC/ABS at 37 °C. Samples were obtained at 4 and 8 h, and the level of pyoverdine within the supernatant fraction was quantified spectrophotometrically as A_{405}. Pyoverdine levels were normalized for growth by dividing A_{405} by the OD_{600} of the cultures. (b) To analyse ETA synthesis, PAO1 was grown in TSB-DC and TSB-DC/ABS at 32 °C. Samples were obtained at 4 and 8 h, and the level of ETA within the supernatant fraction was determined by sandwich ELISA. Levels of ETA were standardized by dividing the amount of ETA in pg ml^{-1} by the OD_{600} of the culture from which the fraction was obtained. Values represent the means ± SEM of three independent experiments. ***, $P<0.001$.

genes. Through initial fractionation experiments, we ruled out the possibility that the mechanism involves a low-molecular-mass protein (<3 kDa) or an unbound peptide that is actively or passively internalized in PAO1 (data not shown). Additional experiments revealed that the potential factor(s) is a high-molecular-mass protein (⩾50 kDa) (Fig. S2). Further analyses indicated that serum albumin contributes substantially to the observed phenomenon, as depletion of albumin from serum eliminated most of the serum-induced enhancement in *pvdS* and *regA* expression (Fig. 7). Similarly, the addition of the therapeutic human serum albumin Albuminar enhanced the expression of both *pvdS* and *regA* at early stages of growth of PAO1 (Fig. 8). Albumin is a complex molecule that contains several cofactors or associated peptides (Foster, 1977; Peters, 1996; Rondeau & Bourdon, 2011). Therefore, the observed effect on *P. aeruginosa* genes may be due to one ore more of these peptides or cofactors. The influence of serum albumin on the production of *P. aeruginosa* virulence factors has been previously reported (Hammond *et al.*, 2010; Kim *et al.*, 2005). Kim *et al.* (2005) showed that serum albumin facilitated the secretion of *P. aeruginosa* type III effector molecules. Analogous to the repression of different *P. aeruginosa* genes by iron, the expression of type III secretion genes, as well as the production of type III effectors, is repressed by calcium (Frank, 1997; Hauser, 2009). Upon its growth in a calcium-deficient medium, *P. aeruginosa* produces maximum levels of type III secretion effectors (Frank, 1997; Hauser, 2009). Kim *et al.* (2005) showed that the addition of serum albumin to a calcium-deficient medium triggered the secretion of intracellularly accumulated type III effector molecules; however, the expression of type III secretion genes was not affected. Serum produced this effect by functioning as a low-affinity calcium-binding protein (Kim *et al.*, 2005). The addition of other low-affinity calcium-binding proteins, including casein, α-lactoalbumin and calreticulin, to the calcium-deficient medium produced a similar phenomenon (Kim *et al.*, 2005). Besides being a low-affinity calcium-binding protein, serum albumin is a low-affinity iron-binding protein (Hider *et al.*, 2010; Silva & Hider, 2009). Therefore, serum albumin may affect the expression of PAO1 genes within its capacity as an iron-binding protein. In serum, iron binds with high affinity to transferrin, a protein which transports iron to different tissues within the body (Hider *et al.*, 2010; Silva & Hider, 2009). Iron may also be transported in serum via ferritin and albumin; however, iron binds to these proteins with less affinity than it binds to transferrin. Additionally, our results clearly rule out the possibility that albumin enhances the expression of different PAO1 genes through its capacity as a low-affinity iron binding protein. Albumin-deficient human serum failed to enhance *pvdS* expression in MM9 (Fig. 9). Further experiments will be conducted to determine if albumin or albumin-associated factor(s) influences the expression of *P. aeruginosa* genes besides *pvdS* and *regA*.

Although the results of our analyses strongly suggest that the influence of serum on the expression of *P. aeruginosa* iron-controlled genes is not directly related to Fur, the specific PAO1 gene or regulatory system through which this regulation is accomplished is not known. It is clear that the effect still requires part of the hierarchical system of iron-regulated genes. In the absence of functional PvdS, which regulates the expression of *regA*, *toxA* and the pyoverdine genes, serum failed to enhance *regA* expression (data not shown). At this time, little is known regarding the relationship between serum and iron-regulated genes in *P. aeruginosa*. One potential link is the previously described virulence and quorum-sensing regulator *vqsR* (Juhas *et al.*, 2004): it was shown by microarray analysis that, in the presence of serum, 113 genes were differentially regulated in the *vqsR* transposon insertion mutant compared with its PAO1 parent strain (Cornelis & Aendekerk, 2004; Juhas *et al.*, 2004).

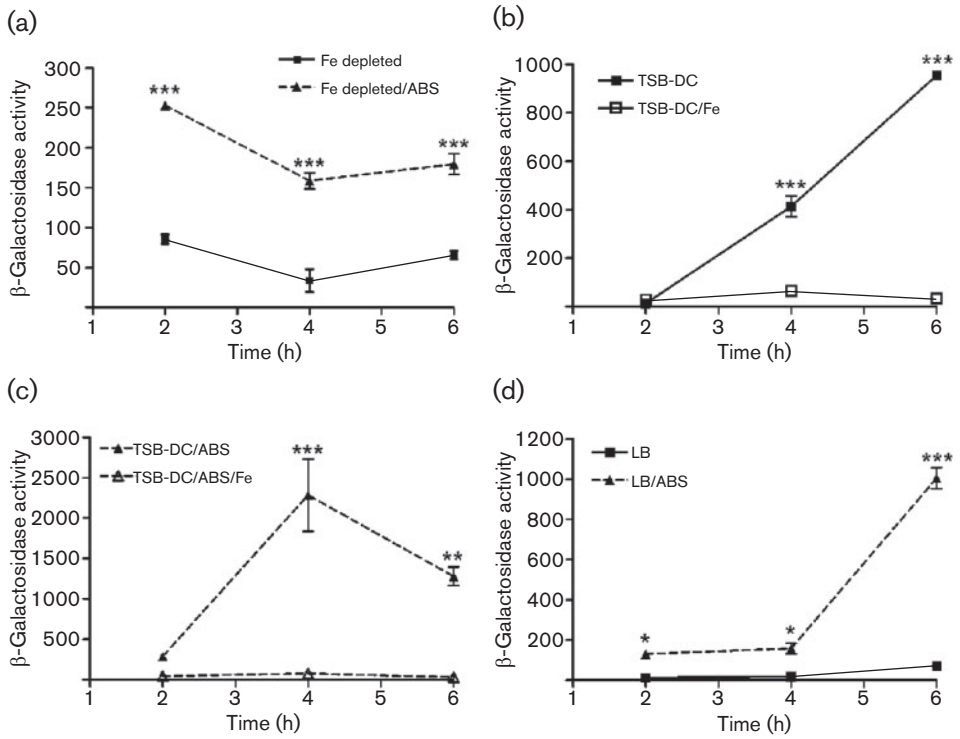

Fig. 5. Serum does not interfere with the repression of *pvdS* expression by iron. PAO1/pPMP220 : : P*pvdS* was grown in the relevant media, and samples were obtained at the indicated time points and the level of *β*-galactosidase activity was determined. Values represent the means ± SEM of three independent experiments. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$. (a) Serum enhances *pvdS* expression when the intracellular pool of iron is depleted. The intracellular pool of iron was depleted by subculturing cells twice in TSB-DC and then adding ABS or continuing the cultures in TSB-DC alone. (b) Iron exogenously added to TSB-DC (high level, 20 μg ml^{-1}) represses *pvdS* expression. (c) High levels of exogenous iron override serum enhancement in *pvdS* expression. (d) Serum enhances *pvdS* expression in PAO1 grown in LB broth, a medium that contains low levels of iron.

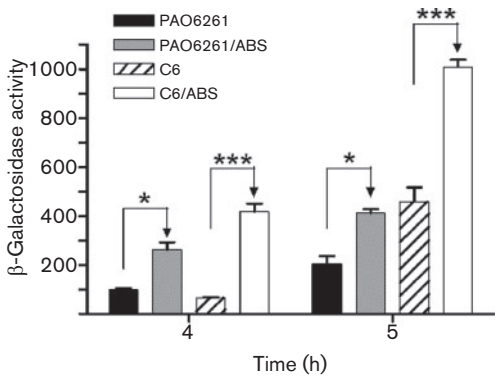

Fig. 6. The effect of serum does not require Fur. The PAO1 *fur* mutant C6 and its parent strain PAO6261 carrying pRL88 were grown in TSB-DC or TSB-DC/ABS and *regA* expression was determined by the levels of *β*-galactosidase activity produced at 4 and 5 h. Values represent the means ± SEM of three independent experiments. ***, $P<0.001$; *, $P<0.05$.

Among the genes whose expression was significantly downregulated in the *vqsR* mutant are 24 genes known to be induced by iron limitation (Juhas *et al.*, 2004). In our study, the expression of 18 of those 24 genes was significantly increased by serum at early stages of growth of PAO1, including genes involved in pyoverdine and pyochelin biosynthesis (Fig. 2, Table 2, Table S1). However, our results also suggest that the observed effect of serum in our study does not occur through *vqsR*. Juhas *et al.* (2004) reported that *vqsR* significantly affects pyocyanin production by PAO1; however, our microarray analysis revealed that serum does not significantly alter the expression of phenazine genes at early- or mid-exponential or late phases of growth (Table S1 and data not shown). We also showed that serum does not affect pyocyanin production by PAO1 (data not shown). Furthermore, our transcriptional analysis revealed that *vqsR* is not among the genes whose expression is significantly affected by serum (Table S1). In contrast, Juhas *et al.* (2004) showed that the addition of serum to a standard medium increased the accumulation of *vqsR* mRNA, suggesting that serum enhances *vqsR* expression. Differences in the findings between our study

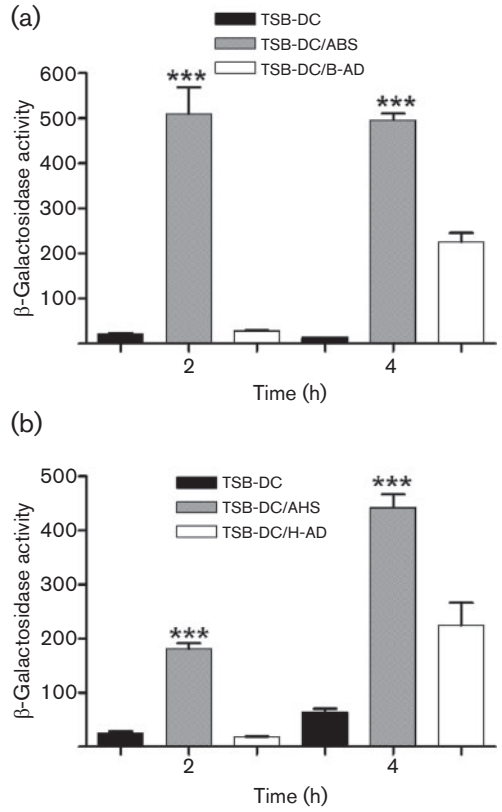

Fig. 7. Albumin depletion abrogates serum enhancement of PAO1 gene expression. DEAE Affi-Gel Blue gel affinity columns (Bio-Rad) were used to deplete ABS (B-AD) and AHS (H-AD) of albumin. PAO1/pPMP220::P*pvdS* was grown in (a) TSB-DC, TSB-DC/ABS or TSB-DC/B-AD; or (b) TSB-DC, TSB-DC/AHS or TSB-DC/H-AD. Samples were obtained at 2 and 4 h and the level of β-galactosidase activity was determined. Values represent the means ± SEM of three independent experiments. ***, $P<0.001$.

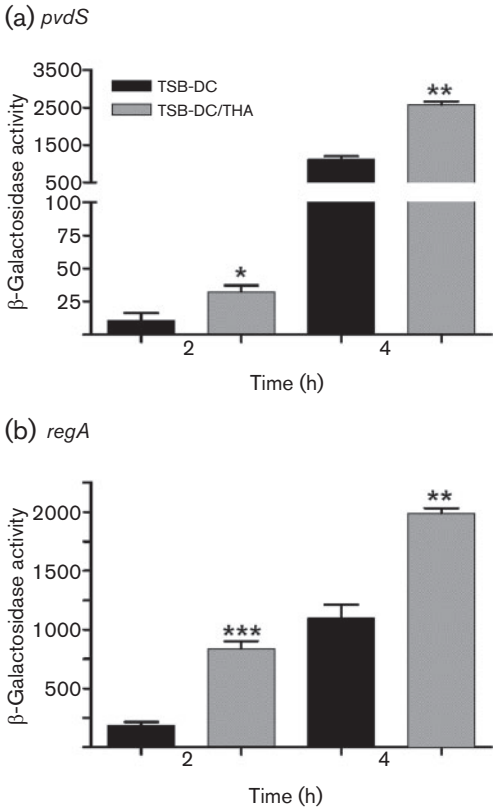

Fig. 8. Therapeutic human albumin (THA) saline solution (Albuminar) enhances *pvdS* expression. PAO1/pPMP220::P*pvdS* (a) and PAO1/pRL88 (b) were grown in either TSB-DC or TSB-DC/THA. Samples were obtained at 2 and 4 h and the level of β-galactosidase activity was determined. Values represent the means ± SEM of three independent experiments. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$.

and that of Juhas *et al.* (2004) are likely due to the different experimental conditions utilized in these studies. Whereas we analysed the effect of serum on PAO1 cultures that were grown to early exponential and early stationary phases in TSB-DC containing 10 % serum, Juhas *et al.* (2004) examined the expression in PAO1 cells from stationary-phase cultures that were grown in RPMI medium containing 10 % serum.

Another potential candidate gene through which serum may accomplish its effect is *PA2384*, which has neither a Fur box nor an IS box in its upstream region, and is less likely to be regulated by Fur (Zheng *et al.*, 2007). The amino-terminal region of the calculated protein encoded by *PA2384* has a weak similarity to the DNA-binding domain of Fur (Zheng *et al.*, 2007). Compared to PAO1, the expression of 71 iron-dependent genes was reduced in a *PA2384* mutant, including 42 related to pyoverdine or pyochelin synthesis and transport, and haem uptake and utilization (Zheng *et al.*, 2007). In addition to *PA2384*, 37 of these same 42 genes were upregulated by the presence of serum in our study (Fig. 2, Table 2, Table S1). Preliminary analyses revealed that, at early phases of growth, serum still enhances pyoverdine production by the PAO1-*PA2384* mutant, while quantitative RT-PCR analysis showed that serum enhanced *pvdS* and *regA* expression in both PAO1 and its *PA2384* mutant (data not shown). Finally, whereas we detect the effect of serum at early phases of growth, most of the *PA2384* target genes are regulated during the stationary phase of growth of PAO1 (Zheng *et al.*, 2007). Thus, serum is unlikely to regulate the expression of PAO1 genes through *PA2384*.

Our recent DNA/gel-shift experiments suggest that, in the early exponential phase, serum enhances the synthesis and/or the activity of a PAO1 DNA-binding protein that specifically binds to the upstream region of *pvdS* (data not shown). Compared with the lysate from PAO1 that was grown in TSB-DC, the lysate from PAO1 that was grown in TSB-DC/ABS produced a more intense specific gel-shift band when it was incubated with the *pvdS* upstream region (data not shown).

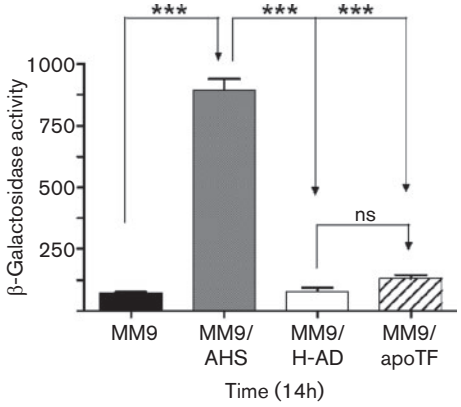

Fig. 9. Serum enhances expression of *pvdS* in medium lacking iron. Cells were grown as described in Fig. 1 and inoculated into MM9 (chemically defined totally iron-deficient medium), MM9/AHS, MM9/H-AD or MM9/apoTF (200 μg apotransferrin ml^{-1}). Cultures were incubated at 37 °C with shaking at 230 r.p.m. for 14 h and the level of β-galactosidase activity was determined. Values represent the means ± SEM of three independent experiments. Statistical significance was determined by one-way ANOVA with Tukey's post test. ***, $P<0.001$; ns, no significant difference.

Thus, the most likely scenario to explain our results is that, at early phases of growth, serum regulates the expression of PAO1 genes through a potential regulator that functions similarly to *PA2384* (i.e. is not regulated by Fur but most of its target genes carry a Fur-binding site). This candidate regulator may be one of the already identified serum-regulated hypothetical transcriptional activators or probable sigma factors (Table 2, Table S1). However, unlike PA2384, the expression of this potential regulator may not be affected by the level of iron in the growth medium.

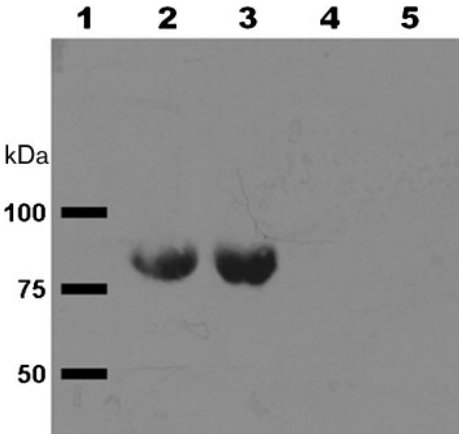

Fig. 10. Transferrin is not present in the albumin-rich serum fraction or in Albuminar. Albumin absorbed from AHS was eluted from the DEAE Affi-Gel Blue gel column. Proteins from each sample were separated by SDS-PAGE, transferred to membrane, and probed with rabbit anti-human transferrin antibody. Lanes: 1, molecular mass standards (band positions indicated by black bars); 2, 30 μg holotransferrin; 3, 30 μg apotransferrin; 4, 50 μg albumin-rich fraction from AHS; 5, 50 μg Albuminar.

ACKNOWLEDGEMENTS

The authors thank Iain Lamont, Keith Poole, Douglas Storey, Michael Vasil, Paolo Visca and Susan West for the strains and plasmids; and Joanna Swickard for critical reading of the manuscript. Strain PW6979 was made available through NIH grant no. P30 DK089507.

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Edited by: M. Whiteley