

# Effect of static growth and different levels of environmental oxygen on *toxA* and *ptxR* expression in the *Pseudomonas aeruginosa* strain PAO1

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Within certain infection sites, such as the lung of cystic fibrosis patients, *Pseudomonas aeruginosa* grows statically under either decreased oxygen tension or anaerobic conditions, a situation that is likely to influence the production of virulence factors. The goal of this study was to determine the effect of static growth under microaerobic (decreased oxygen) and anaerobic conditions on the expression of the *P. aeruginosa* exotoxin A (ETA) gene *toxA* and its positive regulator *ptxR*. Using *toxA-lacZ* and *ptxR-lacZ* fusion plasmids, the level of *toxA* and *ptxR* expression was measured throughout the growth cycle of strain PAO1, which was grown in either iron-deficient or iron-sufficient medium under four different conditions: 20 %-SH (aerobic, shaking), 20 %-ST (aerobic, static), 10 %-ST (microaerobic, static) and 0 %-ST (anaerobic, static). In iron-deficient medium, *toxA* expression was higher under 20 %-ST and 10 %-ST than under 20 %-SH. However, the highest level of *toxA* expression occurred under 0 %-ST. Analysis of ETA protein using sandwich ELISA revealed that at time points between 8 and 24 h of the growth curve, PAO1 produced higher levels of ETA under 0 %-ST than under 20 %-SH. In iron-sufficient medium, *toxA* expression was significantly repressed under all conditions. Additional analyses using PAO1 strains that carry *lacZ* fusions with the *toxA* regulatory genes *regA* and *pvdS* revealed that the expression of *regA* and *pvdS* is reduced rather than increased at 0 %-ST. *ptxR* expression under different conditions paralleled that of *toxA* expression, except that it was repressed by iron under 20 %-SH only. Between 6 and 24 h of growth, and under all conditions, the level of dissolved oxygen (DO) within the PAO1 cultures was sharply reduced. These results suggest that (1) the combined effect of static growth and anaerobic conditions produce a significant increase in *toxA* and *ptxR* expression in PAO1; (2) this effect appears to be unique to *toxA* and *ptxR*, since the level of *regA* and *pvdS* expression was reduced under the same conditions; (3) neither static growth nor anaerobic conditions interfere with the repression of *toxA* expression by iron, although static growth deregulates *ptxR* expression with respect to iron; and (4) the enhanced expression of *toxA* and *ptxR* is not related to the reduced levels of DO in PAO1 cultures.

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## INTRODUCTION

*Pseudomonas aeruginosa* is an opportunistic Gram-negative pathogen that causes serious infections in immunocompromised hosts, including patients with human immunodeficiency virus infections, cancer patients and severely burned patients (Baltch, 1994; Holder, 1993; Pollack, 2000). *P. aeruginosa* is the leading causative agent of chronic lung

infections in cystic fibrosis (CF) patients (Baltch, 1994; Davis *et al.*, 1996). While other micro-organisms such as *Haemophilus influenzae* and *Staphylococcus aureus* also colonize the lungs of CF patients, *P. aeruginosa* becomes the predominant micro-organism as the disease progresses to the chronic stage (Baltch, 1994; Hassett *et al.*, 2002). The defect in chloride secretion in CF results in the accumulation of a stagnant thick mucus within the alveoli of the lung (Baltch, 1994; Jiang *et al.*, 1993). *P. aeruginosa* grows within the thick mucus, producing a persistent infection (Baltch, 1994; Wood *et al.*, 1976). The major survival challenge *P. aeruginosa* faces during this type of infection is the limited supply of oxygen within the mucus (Worlitzsch *et al.*, 2002).

Abbreviations: CF, cystic fibrosis; DO, dissolved oxygen; EO, environmental oxygen; ETA, exotoxin A; Fur, ferric uptake regulator; 20 %-SH, aerobic (20 % EO) with shaking; 20 %-ST, aerobic and static; 10 %-ST, microaerobic (10 % EO) and static; 0 %-ST, anaerobic (0 % EO) and static.

A similar situation also occurs within biofilms that *P. aeruginosa* forms on abiotic and biotic surfaces, including the lungs of CF patients (Costerton *et al.*, 1999; Singh *et al.*, 2002). An oxygen gradient exists within a mature biofilm, where oxygen is usually depleted within 30 µm of the biofilm surface (Xu *et al.*, 1998; Yoon *et al.*, 2002). Such environmental stress is likely to produce several physiological changes in *P. aeruginosa*, including possible variations in the production of virulence factors.

Tissue damage produced during *P. aeruginosa* infections is due to the production of several extracellular and cell-associated virulence factors, including exotoxin A (ETA), elastases, type III secretion proteins, pyocyanin and alginate (Baltch, 1994; Frank, 1997; Govan & Deretic, 1996; Sato & Frank, 2004; Woods & Vasil, 1994). ETA is an ADP-ribosylating enzyme that catalyses the transfer of an NAD moiety onto elongation factor 2, causing cessation of host protein synthesis and cell death (Hamood *et al.*, 2004; Iglewski & Kabat, 1975). Clinical studies have indicated that ETA is an important virulence factor in the pathogenesis of different *P. aeruginosa* infections. For example, Hamood *et al.* (1996a) found that most of the *P. aeruginosa* isolates obtained from patients with wound, urinary tract and respiratory tract infections produced detectable levels of ETA. In addition, ETA antibodies have been detected in the sera of CF patients infected with *P. aeruginosa* (Hollsing *et al.*, 1987; Jagger *et al.*, 1982; Pollack *et al.*, 1976). Increasing levels of IgG antibodies to *P. aeruginosa* LPS and ETA in CF patients are usually associated with a poor prognosis (Moss *et al.*, 1986). Furthermore, the detection of *toxA* mRNA in the sputum samples obtained from CF patients indicates that *toxA* is transcribed by *P. aeruginosa* within the lungs of these patients (Raivio *et al.*, 1994; Storey *et al.*, 1998). Besides the clinical studies, several animal studies using purified ETA or ETA-deficient mutants have demonstrated that ETA plays a critical role in the virulence of *P. aeruginosa* (Fogle *et al.*, 2002; Matsumoto *et al.*, 1999; Nicas & Iglewski, 1985; Rahme *et al.*, 1995).

ETA production by *P. aeruginosa in vitro* is regulated by several environmental factors, including growth temperature, concentration of iron in the growth medium, and the presence of certain nucleotides and amino acids in the growth medium (Hamood *et al.*, 2004; Liu, 1973). The most extensively analysed of these factors is iron, which represses the transcription of the ETA gene, *toxA* (Hamood *et al.*, 2004; Lory, 1986). Maximum levels of *toxA* transcription are usually detected when *P. aeruginosa* is grown in iron-deficient medium (Frank & Iglewski, 1988; Grant & Vasil, 1986; Hamood *et al.*, 2004; Lory, 1986). The complicated process of ETA production by *P. aeruginosa* also involves several positive regulatory genes, including *regA*, *ptxR* and *pvdS* (Hamood *et al.*, 2004). The *regA* locus is essential for *toxA* expression in *P. aeruginosa*; no *toxA* mRNA was detected in a *regA* isogenic mutant of *P. aeruginosa* (Hamood *et al.*, 2004; Wick *et al.*, 1990). However, the exact mechanism through which *regA* regulates *toxA* expression is

not completely defined. The 29 kDa RegA protein encoded by *regA* neither binds to the *toxA* upstream region nor carries significant homology to other prokaryotic transcriptional activators (Hamood & Iglewski, 1990; Hamood *et al.*, 2004; Raivio *et al.*, 1996). The *ptxR* gene encodes PtxR, a 34 kDa protein that belongs to the LysR family of transcriptional activators (Hamood *et al.*, 1996b, 2004). The presence of a *ptxR* plasmid in *P. aeruginosa* enhances *toxA* expression by four- to fivefold (Hamood *et al.*, 1996b, 2004). Available evidence suggests that *ptxR* regulates *toxA* expression through *regA*, although unlike *regA*, *ptxR* is not essential for *toxA* expression (Hamood *et al.*, 1996b, 2004). The alternative sigma factor PvdS was originally described as a transcriptional activator of the pyoverdine synthesis genes (Cunliffe *et al.*, 1995; Hamood *et al.*, 2004). PvdS specifically binds to a DNA sequence element, the iron-starvation (IS) box, within the upstream region of the pyoverdine synthesis genes *pvdE* and *pvdF* (Wilson *et al.*, 2001). PvdS is also required for the expression of *toxA*, *regA* and *ptxR* (Beare *et al.*, 2003; Hamood *et al.*, 2004). Iron negatively regulates the expression of several *P. aeruginosa* genes through the ferric uptake regulator (Fur; Hamood *et al.*, 2004; Vasil & Ochsner, 1999), including the siderophore regulatory genes (*pchR* and *pvdS*), *toxA*, *regA* and *ptxR* (Hamood *et al.*, 2004; Vasil & Ochsner, 1999). Fur regulates *pchR* and *pvdS* by specifically binding to the Fur-binding box in their upstream regions (Ochsner *et al.*, 1995). Available evidence suggests that Fur regulates the expression of *toxA*, *regA* and *ptxR* through *pvdS* (Barton *et al.*, 1996). Based on the analysis of several PAO1 *fur* mutants, Barton *et al.* (1996) proposed that Fur regulates *toxA* and *regA* through *pvdS* under microaerobic conditions. Vasil *et al.* (1998) suggested a similar scenario for the regulation of *ptxR* expression by Fur.

Despite extensive analyses of *toxA* expression, our knowledge regarding the effect of environmental oxygen (EO) on *toxA* expression throughout the growth cycle of *P. aeruginosa* is still incomplete. The standard protocol to examine *toxA* expression *in vitro* involves growing *P. aeruginosa* cultures at 32 °C with maximum aeration (shaking the culture flasks at 250 r.p.m. under aerobic conditions) (Hamood *et al.*, 2004; Wick *et al.*, 1990). However, as demonstrated by several studies, the conditions within infection sites, such as the lung alveoli of CF patients or infected wounds, are likely to be either hypoxic (microaerobic) or anaerobic (Hohn *et al.*, 1976; Worlitzsch *et al.*, 2002; Xu *et al.*, 1998; Yoon *et al.*, 2002). Therefore, in this study, we tried to determine if static growth and different levels of EO affect *toxA* and *ptxR* expression throughout the growth cycle of *P. aeruginosa*, and if these different levels interfere with the negative regulation of *toxA* and *ptxR* expression by iron.

## METHODS

**Bacterial strains, plasmids and growth media.** The *P. aeruginosa* prototrophic strain PAO1 (Holloway *et al.*, 1979) was utilized

to examine *toxA* expression and ETA production. To examine the effect of *pvdS* on *toxA* expression, we utilized the mutant PAO1::*pvdS*, which carries a specific deletion within *pvdS*, as described by Cunliffe *et al.* (1995). The expression of *regA* and *pvdS* was examined using PAO1 clones 6424 and 2812, respectively (Bailey & Manoil, 2002). The clones were obtained from the UWGC Mutant Library (Department of Medicine, University of Washington, Seattle, WA, USA; <http://www.genome.washington.edu/UWGC/pseudomonas/index.cfm>). In 6424, the region that encodes the first 125 aa of RegA is fused in-frame with the  $\beta$ -galactosidase gene (Jacobs *et al.*, 2003). In 2812, the region that encodes the first 116 aa of PvdS is fused in-frame with the  $\beta$ -galactosidase gene (Jacobs *et al.*, 2003). The previously described plasmid pSW228, which carries a *toxA-lacZ* translational fusion, was utilized to examine *toxA* expression in PAO1. In this plasmid, 760 bp of the *toxA* upstream region plus the region that encodes the first seven amino acids of ETA is fused in-frame with the  $\beta$ -galactosidase gene (West *et al.*, 1994).

For general growth experiments, including preparation of overnight cultures, plasmid DNA extraction and electroporation, PAO1 was grown in Luria-Bertani (LB) broth (Miller, 1972). For aerobic and microaerobic conditions, PAO1 was grown in iron-deficient medium (TSB-DC) or iron-sufficient medium (TSB-DC plus iron). TSB-DC is a chelexed trypticase soy broth dialysate containing 1% (v/v) glycerol and 0.5 M monosodium glutamate (Ohman *et al.*, 1980). Iron as FeCl<sub>3</sub> (10 mg Fe<sup>3+</sup> ml<sup>-1</sup>) was added to TSB-DC at a concentration of 25  $\mu$ g Fe<sup>3+</sup> ml<sup>-1</sup> (Frank & Iglewski, 1988; Hamood *et al.*, 1996b). For anaerobic growth, cells were grown in TSB-DC supplemented with 1% potassium nitrate (KNO<sub>3</sub>) as a terminal electron acceptor (Hasset, 1996). To maintain the plasmids in PAO1, carbenicillin was added to the growth medium at a concentration of 300  $\mu$ g ml<sup>-1</sup>.

**Growth conditions.** PAO1 containing different plasmids was grown overnight in LB broth under 20% EO with shaking (20%-SH) at 37 °C. A 1.5 ml aliquot of the culture was pelleted, washed, and resuspended in 300  $\mu$ l TSB-DC medium. The resuspended cells were inoculated into 100 ml fresh TSB-DC medium to an initial OD<sub>600</sub> of 0.03–0.05. Aliquots (5 ml) of the inoculated medium were then dispensed into 125 ml flasks, one for each time point and condition. Flasks were incubated at 32 °C aerobically in a shaking (250 r.p.m.) water bath (20%-SH) or in a nonshaking incubator (20%-ST). For microaerobic static conditions (10%-ST), flasks for each time point were sealed into individual GasPak Jars (Becton Dickinson) with Campy Pak Plus envelopes (Becton Dickinson), which are designed to generate the microaerobic atmosphere (10% EO), and incubated in the nonshaking 32 °C incubator. For anaerobic static conditions (0%-ST), the resuspended cells were inoculated into 100 ml TSB-DC containing 1% KNO<sub>3</sub> to an OD<sub>600</sub> of 0.03–0.05. Aliquots (5 ml) of the diluted culture were dispensed into 5 ml polystyrene round-bottom tubes (Falcon; BD Sciences), leaving a very small space between the surface of the culture and the cap of the tube. Anaerobic conditions were generated using Oxyrase For Broth (Oxyrase, Inc.), which contains the Oxyrase Enzyme System and a blend of substrates to maximize Oxyrase activity, following the manufacturer's recommendations. Oxyrase For Broth decreases the oxygen concentration within aerobic cultures to less than 10 parts per billion (0% EO) in 30 min, and maintains these conditions for at least 16 days. A methylene-blue anaerobic indicator strip that changes to colourless in oxygen-free medium (Becton Dickinson) was included in a control tube. Tightly closed tubes were incubated in the nonshaking 32 °C incubator. Throughout the 24 h growth cycle, flasks or tubes for each time point were removed from their incubation conditions, and samples of the cultures were obtained for analysis. Each growth curve experiment was repeated three times.

**$\beta$ -Galactosidase assay.** For each growth condition and time point throughout the growth cycle, duplicate samples were obtained, and cells were pelleted for the  $\beta$ -galactosidase assay, which was performed as described by Stachel *et al.* (1985). Briefly, pelleted cells were resuspended in 600  $\mu$ l *lacZ* buffer (0.06 M Na<sub>2</sub>HPO<sub>4</sub>/0.04 M NaH<sub>2</sub>PO<sub>4</sub>/0.01 M KCl/0.001 M MgSO<sub>4</sub>; 0.05 M  $\beta$ -mercaptoethanol was added prior to use). A 100  $\mu$ l sample was removed to determine the cell density by measuring the OD<sub>600</sub>. Samples were lysed with chloroform and SDS, and the level of  $\beta$ -galactosidase activity was determined as described by Stachel *et al.* (1985). The following formula was utilized to calculate the units of  $\beta$ -galactosidase activity:  $(A_{420} \times 10^3)/(OD_{600} \times t)$ , in which  $t$  is incubation time (min) (Stachel *et al.*, 1985).

**Sandwich ELISA.** The assay was done as described by Coligan *et al.* (2001), using 96-well microtitre immunoassay plates (Immulon 2HB; Dynex Technologies). Throughout the assay, the plates were washed with PBST buffer (0.02% v/v, Tween 20 in phosphate-buffered saline). Each well was coated with 100  $\mu$ l diluted goat-anti-ETA antibody (0.25  $\mu$ g ml<sup>-1</sup> in 100 mM Na<sub>2</sub>HCO<sub>3</sub>) (List Biologicals) overnight at 4 °C. The plates were washed, and treated with bovine serum albumin, 1 mg ml<sup>-1</sup> in PBST, for 1 h at 37 °C to block non-specific binding sites. The plates were then washed twice, and incubated with different supernatant fractions (100  $\mu$ l per well) for 1 h at room temperature. As a standard, we utilized several dilutions (2–62.5 pg  $\mu$ l<sup>-1</sup> in PBST) of purified ETA (MP Biomedicals). The plates were washed six times, and incubated with rabbit-anti-ETA (100  $\mu$ l per well) (Fogle *et al.*, 2002), which was diluted in PBST, for 1 h at room temperature. The plates were then washed six times, and incubated with goat-anti-rabbit IgG conjugated to horseradish peroxidase (Sigma-Aldrich) for 1 h at room temperature. The plates were then washed six times, and incubated with 100  $\mu$ l substrate solution per well (ImmunoPure TMB Substrate; Pierce Biotechnology) at 37 °C for 5 min. The reaction was stopped by adding 100  $\mu$ l 2 M H<sub>2</sub>SO<sub>4</sub> per well. The absorbance was read at 450 nm using an ELISA plate reader (Bio-Tek Instruments). The values were standardized by dividing the amount of ETA (pg  $\mu$ l<sup>-1</sup>) from each supernatant fraction by the OD<sub>600</sub> of the culture from which that fraction was obtained.

**Measuring dissolved oxygen.** The level of dissolved oxygen (DO) within each culture at each time point was determined using the Dissolved Oxygen Measuring System (Instech), as recommended by the manufacturer. Basically, a flask containing uninoculated TSB-DC medium was incubated together with the PAO1 cultures under the tested conditions. At each time point, the machine was standardized by placing 1 ml uninoculated TSB-DC in the measuring chamber, and the reading was set at 100%. The uninoculated TSB-DC was then replaced with the PAO1 culture, and the percentage of DO was recorded.

**Statistical analysis.** Statistics were calculated using InStat (Graph Pad Software). ANOVA was used to determine significant differences in the expression of *toxA* and *ptxR* among the various conditions.

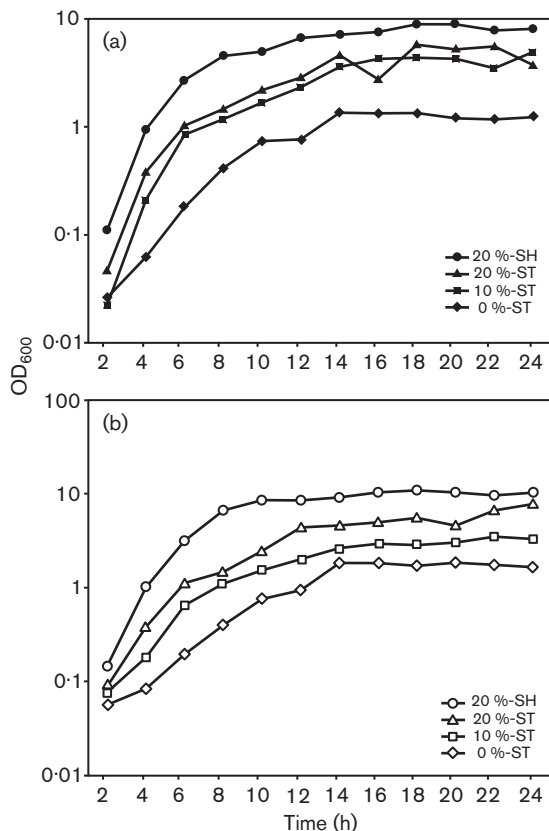
## RESULTS

### Comparing the normal growth cycle of PAO1 under different oxygen levels

In most research laboratories, *P. aeruginosa* is grown under maximum aeration – aerobic conditions (20% environmental oxygen, EO) with vigorous shaking (250 r.p.m.) – to examine the expression of its different genes *in vitro*. However, in several infection sites, such as the thick mucus

in the lung alveoli of the CF patient, *P. aeruginosa* grows in a static state, with a significantly lower level of EO (Worlitzsch *et al.*, 2002). Therefore, to examine the effect of static growth and EO on *toxA* and *ptxR* expression under conditions that more closely resemble those *in vivo*, we grew PAO1 under the following conditions: aerobic/static (20 %-ST), micro-aerobic/static (10 %-ST) and anaerobic/static (0 %-ST). We included the aerobic/shaking condition (20 %-SH) for comparison, since this was the condition under which we grew PAO1 in all of our previous analyses of *toxA* and *ptxR* expression (Hamood *et al.*, 2004).

We analysed the growth cycle of PAO1 under the different EO levels described above, in both iron-deficient and iron-sufficient media. All cultures were standardized to an OD<sub>600</sub> of 0.03–0.05 at the time of inoculation (zero time). Samples were obtained every 2 h, and the OD<sub>600</sub> was determined. As shown in Fig. 1, throughout the growth cycle under 20 %-ST and 10 %-ST, we detected comparable growth of PAO1. For the four tested conditions, least growth was detected



**Fig. 1.** Growth curves of PAO1 grown in shaking or static conditions under different levels of EO, and in (a) iron-deficient and (b) iron-sufficient medium. Cells were grown as described in Methods. Samples were obtained every 2 h, and the OD<sub>600</sub> was determined. Circles represent growth under 20 %-SH; triangles, 20 %-ST; squares, 10 %-ST; and diamonds, 0 %-ST. The graph shown is representative of three independent experiments.

under 0 %-ST, while the highest level of growth was seen under 20 %-SH (Fig. 1). Under all conditions, the growth of PAO1 was slightly enhanced in the presence of iron (Fig. 1b). Under all the different conditions, cells appeared to reach stationary phase at 12–14 h. No major change in growth was detected after this time (Fig. 1).

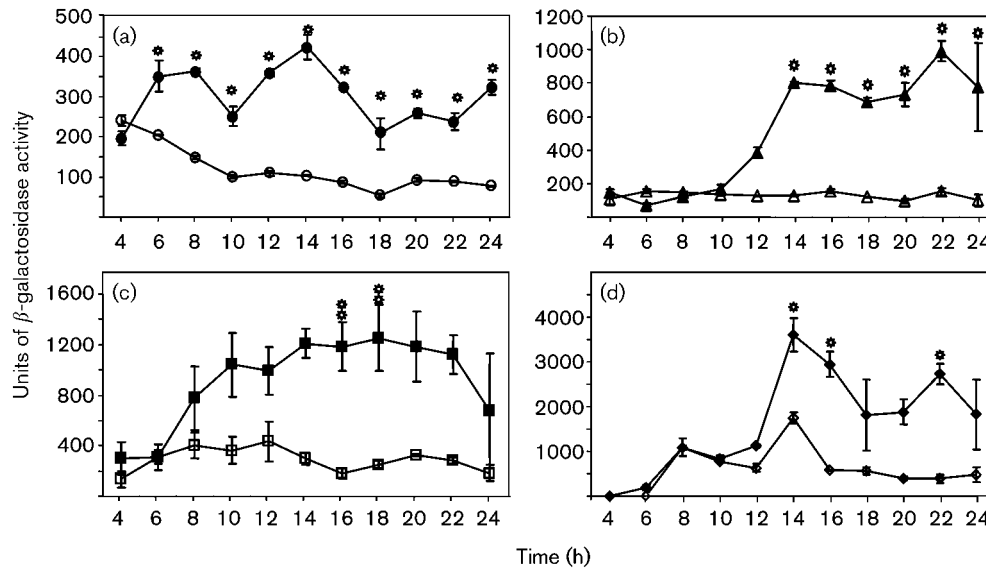
### Effect of static growth and EO level on *toxA* expression in PAO1

Having established the consistency of the growth cycle of PAO1, we then analysed the effect of the different conditions on *toxA* expression. Plasmid pSW228 was utilized to examine *toxA* expression (West *et al.*, 1994). This plasmid was generated from the promoterless *lacZ* cloning vector pSW205, which replicates stably in *P. aeruginosa* (West *et al.*, 1994). PAO1 carrying pSW205 or pSW228 was grown in either iron-deficient or iron-sufficient medium under the four conditions for 24 h at 32 °C. Samples were obtained every 2 h, and the level of  $\beta$ -galactosidase activity was determined as described by Stachel *et al.* (1985). The growth rate (OD<sub>600</sub>) is incorporated into the formula for calculating the units (U) of  $\beta$ -galactosidase activity (Stachel *et al.*, 1985); thus, any remaining growth-related bias is compensated. Growth curves similar to those in Fig. 1 were obtained with PAO1 carrying pSW228 or pSW205 (data not shown). PAO1 carrying pSW205 produced no detectable level of  $\beta$ -galactosidase activity under any condition of growth (data not shown).

**20 %-SH.** In iron-deficient medium, *toxA* expression followed a biphasic curve in which the first peak was detected between 6 and 8 h, while the second peak occurred at 14 h (Fig. 2a). In iron-sufficient medium, *toxA* expression showed no specific features, and the level of expression was significantly ( $P < 0.001$ ) lower than that produced in iron-deficient medium throughout the growth cycle (Fig. 2a).

**20 %-ST.** In both iron-deficient and iron-sufficient media, comparable levels of *toxA* expression were detected between the 4 and 10 h time points (Fig. 2b). After that time, *toxA* expression increased sharply until the 14 h time point, and then levelled off in iron-deficient medium, but remained unchanged in iron-sufficient medium (Fig. 2b). The level of *toxA* expression in iron-deficient medium was significantly ( $P < 0.001$ ) higher than that in iron-sufficient medium from 12 to 24 h (Fig. 2b).

**10 %-ST.** The pattern of *toxA* expression was basically similar to that under 20 %-ST, except that the increase in the level of the expression was detected at the 8 h time point (Fig. 2c). At the 16 and 18 h time points, the level of *toxA* expression was significantly higher ( $P < 0.05$ ) in iron-deficient medium than in iron-sufficient medium (Fig. 2c).



**Fig. 2.** Effect of shaking or static conditions under different levels of EO on *toxA* expression throughout the growth cycle of PAO1. PAO1 carrying the *toxA-lacZ* fusion plasmid pSW228 was grown as described in Methods. Duplicate samples were obtained every 2 h, and the level of  $\beta$ -galactosidase activity was determined. (a) 20%-SH, (b) 20%-ST, (c) 10%-ST, (d) 0%-ST. Filled symbols, iron-deficient medium; open symbols, iron-sufficient medium. Values represent the means of three independent experiments ( $\pm$  SEM). One asterisk,  $P < 0.001$ ; two asterisks,  $P < 0.05$ .

**0 %-ST.** Similar to 20 %-ST, comparable levels of *toxA* expression were detected in iron-deficient and iron-sufficient media from 4 h through to 10 h, (Fig. 2d). In iron-deficient medium, two peaks of *toxA* expression were detected, a major one at 14 h, and a smaller one at 22 h (Fig. 2d). In iron-sufficient medium, a single peak of *toxA* expression was detected at the 14 h time point (Fig. 2d). At several time points, *toxA* expression in iron-deficient medium was significantly ( $P < 0.001$ ) higher than that in iron-sufficient medium (Fig. 2d).

### Comparison of *toxA* expression under the different growth conditions

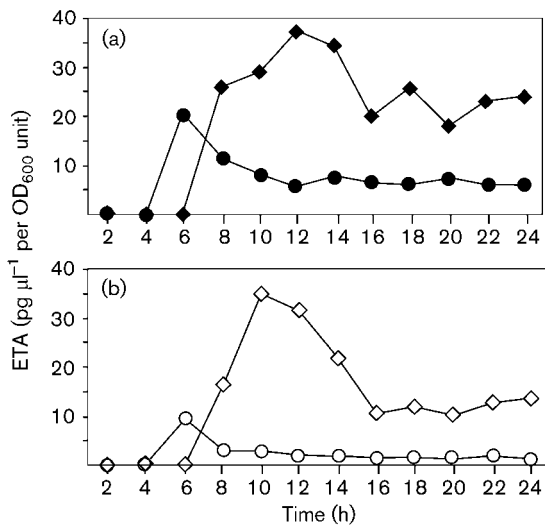
Based on the analysis of *toxA* expression under the different growth conditions, we made the following observations. (1) In iron-deficient medium, static growth and anaerobic conditions enhance *toxA* expression in PAO1 (Fig. 2). Under 20 %-SH, the level of *toxA* expression ranged from 200 to 400 U  $\beta$ -galactosidase activity (Fig. 2a). The range increased to 200–1000 U under 20 %-ST, 250–1200 U under 10 %-ST, and 200–3600 U under 0 %-ST (Fig. 2b–d). The most significant increases in *toxA* expression occurred under 0 %-ST in both iron-deficient and iron-sufficient media (at least ninefold higher than that under 20 %-SH) (Fig. 2). (2) Between the 12 and 22 h time points, neither static growth nor anaerobic conditions abolished the repression of *toxA* expression by iron (Fig. 2). However, at earlier stages of growth (4–10 h) under 20 %-ST and 0 %-ST, *toxA* expression appeared to be deregulated with respect to iron (Fig. 2b, d). (3) As previously described, two peaks of *toxA*

expression were detected, at 6–8 h and 14 h of growth, under 20 %-SH, and in iron-deficient medium only (Hamood *et al.*, 2004). Under both 20 %-ST and 10 %-ST, *toxA* expression was characterized by an initial increase that plateaued throughout the remainder of the growth cycle (Fig. 2b, c). Under 0 %-ST, two peaks of *toxA* expression were detected, but later in the growth cycle, at 14 and 22 h (Fig. 2d). In addition, the 14 h peak appeared to be present even in iron-sufficient medium, although at a lower level (Fig. 2d).

Maximum production of ETA occurs *in vitro* when *P. aeruginosa* is grown at 32 °C (Hamood *et al.*, 2004). Accordingly, the above-described experiments were conducted at 32 °C. However, the temperature within the lung alveoli (including the lung of CF patients) is likely to be closer to the core temperature of the body, which is 37 °C (Jessen, 2001). Therefore, we tried to determine if the increase in *toxA* expression under 0 %-ST occurs at 37 °C. PAO1/pSW228 was grown under 20 %-SH and 0 %-ST at 32 and 37 °C. In iron-deficient medium, and at both temperatures, *toxA* expression was higher under 0 %-ST than under 20 %-SH (data not shown).

### Effect of anaerobic conditions on ETA production by PAO1

The above results showed that the most significant increase in *toxA* expression occurs under 0 %-ST. Thus, we tried to determine if, similar to *toxA* expression, ETA production by PAO1 is enhanced under 0 %-ST. Cells were grown under either 20 %-SH or 0 %-ST for 24 h, and samples were



**Fig. 3.** Amount of ETA in the supernatant fraction of PAO1 grown in (a) iron-deficient, and (b) iron-sufficient medium, under either 20%-SH and 0%-ST, as determined by sandwich ELISA (Methods). Values were standardized by dividing the amount of ETA (pg µl<sup>-1</sup>) in each fraction by the OD<sub>600</sub> of the culture from which the fraction was obtained. 20%-SH, circles; 0%-ST, diamonds; filled symbols, iron-deficient medium; open symbols, iron-sufficient medium. Values represent the mean of three replicates from a single experiment. Similar results were obtained from additional experiments.

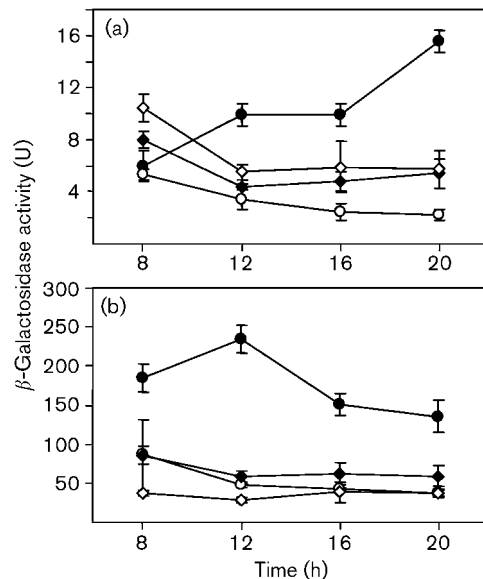
obtained every 2 h. The supernatant fractions were isolated, and the amount of ETA in each fraction was determined by sandwich ELISA as described (Methods; Coligan *et al.*, 2001). In iron-deficient medium, and between the 8 and 24 h time points, PAO1 produced considerably higher amounts of ETA under 0%-ST than under 20%-SH (Fig. 3a). In iron-sufficient medium, and throughout the growth cycle, PAO1 produced very low levels of ETA under 20%-SH (Fig. 3b). In comparison, higher levels of ETA were produced under 0%-ST (Fig. 3b). These results confirm our *toxA-lacZ* transcriptional analyses, and indicate that the growth of PAO1 under 0% EO enhances ETA production. With respect to iron, and similar to *toxA* transcription, we detected no major difference in the level of ETA protein within the supernatant of PAO1 that was grown under 0%-ST at the 10 and 12 h time points (Fig. 3). After 14 h, the amount of ETA in the supernatant of PAO1 grown in iron-deficient medium was considerably higher than that in iron-sufficient medium (Fig. 3).

Comparison of the results in Figs 2 and 3 shows that in iron-deficient medium under 0%-ST, the maximum level of *toxA* transcription occurs at 14 h, while the maximum level of ETA production occurs at 12 h. The simplest explanation for this observation is that at specific time points in these separate experiments, the growth rate of PAO1 is not necessarily the same. At the two time points

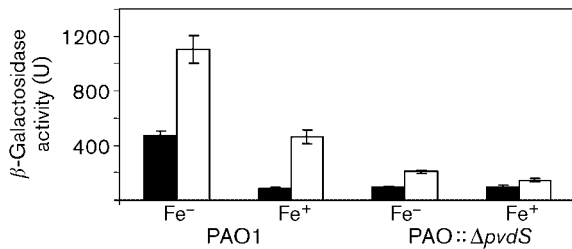
described above, PAO1 reached a comparable growth index of OD<sub>600</sub> 1.35–1.40.

### Effect of anaerobic conditions on *pvdS* and *regA* expression in PAO1

ETA production by *P. aeruginosa* is a multi-layered process that includes several regulators. Therefore, the elimination of EO (0%-ST) may enhance *toxA* expression through one of these regulators, such as the alternative sigma factor PvdS and/or the *toxA* transcriptional activator RegA (Hamood *et al.*, 2004). PvdS is important for the production of ETA by *P. aeruginosa*: a *pvdS* deletion mutant produced significantly lower levels of *toxA* mRNA (Ochsner *et al.*, 1996). Similarly, RegA is essential for *toxA* expression in *P. aeruginosa*. Several previous studies showed that both positive and negative regulation of *toxA* expression occur through *regA* (Hamood *et al.*, 2004). We determined that the growth of PAO1 under anaerobic conditions affects the expression of *regA* and *pvdS*. This was done using *P. aeruginosa* clones 6424 and 2812, which carry chromosomal *regA-lacZ* and *pvdS-lacZ* translational fusions, respectively, both generated from PAO1 (Jacobs *et al.*, 2003). Cells were grown as described above, and samples were obtained at 8, 12, 16 and 20 h. In iron-deficient medium, *regA* expression under 20%-SH was higher than that under 0%-ST at 12, 16 and 20 h (Fig. 4a).



**Fig. 4.** Effect of EO on *regA* and *pvdS* expression in PAO1. PAO1 carrying either a chromosomal *regA-lacZ* translational fusion (6424), or a chromosomal *pvdS-lacZ* translational fusion (2812), was grown as described in Methods. Duplicate samples were obtained at the 8, 12, 16, and 20 h time points, and the level of β-galactosidase activity was determined. (a) *regA* expression in 6424. (b) *pvdS* expression in 2812. 20%-SH, circles; 0%-ST, diamonds. Filled symbols indicate iron-deficient medium; open symbols indicate iron-sufficient medium. Values represent the means of three independent experiments (± SEM).



**Fig. 5.** Levels of *toxA* expression in PAO1/pSW228 and PAO::*pvdS*/pSW228 grown in iron-deficient and iron-sufficient media under 20%-SH (black columns) and 0%-ST (white columns). Cells were grown as described in Methods, and the level of  $\beta$ -galactosidase activity was determined. Samples were obtained at the 16 h time point. Similar results were obtained at other time points. Each value represents the mean of three independent experiments ( $\pm$  SEM).

However, in iron-sufficient medium, no major differences were detected (Fig. 4a). In addition, *pvdS* expression was considerably higher under 20%-SH than under 0%-ST at all time points in iron-deficient medium only (Fig. 4b). These results indicate that the growth of PAO1 under 0%-ST decreases rather than increases *regA* and *pvdS* expression. This suggests that, despite the dependence of *toxA* on *pvdS* and *regA* for its expression under 20%-SH conditions, it is regulated differently under anaerobic conditions.

Although the enhancement in *toxA* expression may not occur through *regA* or *pvdS*, it may still require a functional *pvdS* or *regA*, or both (i.e. it is not completely independent of these genes). To examine this possibility with respect to *pvdS*, we determined the level of *toxA* expression in the *pvdS* deletion mutant PAO::*pvdS* (Cunliffe *et al.*, 1995). In iron-deficient medium, and in comparison with PAO1/pSW228, the level of *toxA* expression in PAO::*pvdS*/pSW228 was reduced under both 20%-SH and 0%-ST by about fivefold (Fig. 5). This indicates that PvdS is required for *toxA* expression under both conditions. Despite this reduction, however, the level of *toxA* expression in PAO::*pvdS*/pSW228 under 0%-ST was twofold higher than that produced under 20%-SH (Fig. 5). This difference paralleled that detected in PAO1/pSW228 (twofold increase under 0%-ST) (Fig. 5). Even in iron-sufficient medium, the increase in *toxA* expression under 0%-ST (in comparison with 20%-SH) was about twofold for PAO::*pvdS*/pSW228, and fourfold for PAO1/pSW228 (Fig. 5). These results suggest that the increase in *toxA* expression due to anaerobic conditions is *pvdS* independent. However, maximum *toxA* expression still requires PvdS.

### Effect of static growth and EO level on *ptxR* expression in PAO1

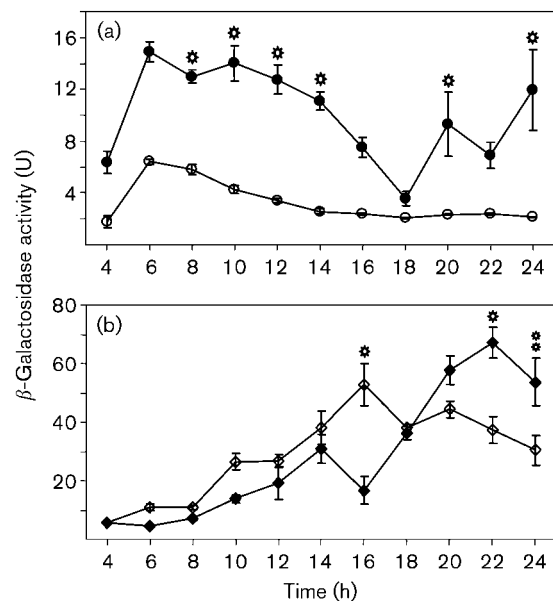
One of the *toxA* transcriptional activators is PtxR. To determine if the pattern of *ptxR* expression under the above-described conditions may be similar to that of *toxA*, we utilized the previously described fusion plasmid pJAC24,

in which the *ptxR* upstream region plus the region that encodes the first 55 aa of PtxR is fused in-frame with the  $\beta$ -galactosidase gene, to examine *ptxR* expression (Colmer & Hamood, 1998). PAO1 containing pJAC24 was grown as described above for PAO1/pSW228. The presence of pJAC24 did not alter the growth curve for PAO1 (data not shown).

**20%-SH.** In iron-deficient medium, *ptxR* expression was detected at the 4 h time point, reached a peak at the 6 h time point, gradually declined until the 18 h time point, and then increased again to the end of the growth cycle (Fig. 6a). In iron-sufficient medium, *ptxR* expression showed no major variations (Fig. 6a). Overall, *ptxR* expression was significantly ( $P < 0.001$ ) lower in iron-sufficient medium than in iron-deficient medium at several time points (Fig. 6a).

**20%-ST and 10%-ST.** *ptxR* expression showed no specific features in iron-deficient or iron-sufficient medium (data not shown). The level of *ptxR* expression in iron-deficient medium was not significantly different from that in iron-sufficient medium (data not shown).

**0%-ST.** Between the 4 and 14 h time points, *ptxR* expression in iron-deficient medium paralleled, but was lower



**Fig. 6.** Effect of shaking or static conditions under different levels of EO on *ptxR* expression throughout the growth cycle of PAO1. PAO1 carrying the *ptxR*-*lacZ* fusion plasmid pJAC24 was grown as described in Methods. Duplicate samples were obtained every 2 h, and the level of  $\beta$ -galactosidase activity was determined. (a) 20%-SH, (b) 0%-ST. Filled symbols, iron-deficient medium; open symbols, iron-sufficient medium. Values represent the means of three independent experiments ( $\pm$  SEM). One asterisk,  $P < 0.001$ ; two asterisks,  $P < 0.05$ .

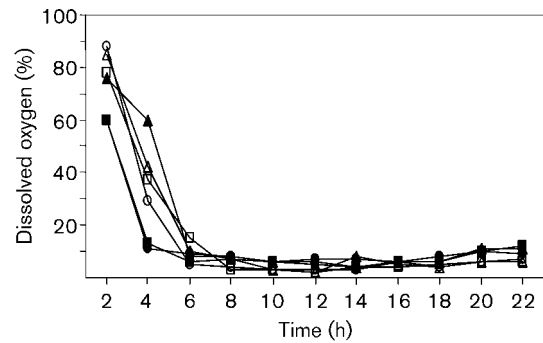
than, that in iron-sufficient medium (Fig. 6b). *ptxR* expression in iron-sufficient medium reached a peak at 16 h, and then declined to the end of the growth cycle, whereas in iron-deficient medium, *ptxR* expression reached a peak at 14 h, declined at 16 h, but then increased to a second peak at 22 h (Fig. 6b). At 16 h, *ptxR* expression in iron-sufficient medium was significantly ( $P < 0.001$ ) higher than that in iron-deficient medium (Fig. 6b). This difference was reversed at the 22 h ( $P < 0.001$ ) and 24 h ( $P < 0.05$ ) time points (Fig. 6b).

### Comparison of *ptxR* expression under the different growth conditions

Analysis of *ptxR* expression provided us with the following observations. (1) Under all conditions, *ptxR* is expressed in PAO1 at a low level. For example, under 20 %-SH at different time points, the level of *ptxR* expression ranged from only 2 to 14 U  $\beta$ -galactosidase activity (Fig. 6a). In contrast, *toxA* expression ranged from 200 to 400 U  $\beta$ -galactosidase activity (Fig. 2a). The highest level of *ptxR* expression was 67 U, which was detected at 22 h under 0 %-ST (Fig. 6b), while *toxA* expression reached 3600 U  $\beta$ -galactosidase activity (Fig. 2d). This low level of *ptxR* expression, which we have previously reported, is a characteristic feature of genes encoding LysR-type proteins (Colmer & Hamood, 1999). (2) *ptxR* expression is negatively regulated by iron under 20 %-SH only; in iron-sufficient medium, *ptxR* expression was two- to sixfold lower than that in iron-deficient medium (Fig. 6a). This difference was eliminated by static growth (Fig. 6; data not shown). At several time points under 20 %-ST, *ptxR* expression in iron-sufficient medium was higher than that in iron-deficient medium (data not shown). Similarly, between 6 and 16 h under 0 %-ST, *ptxR* expression in iron-sufficient medium was higher than that in iron-deficient medium (Fig. 6b). *ptxR* expression in iron-deficient medium under 0 %-ST was significantly higher than that in iron-sufficient medium, only from 20 to 24 h (Fig. 6b). These results suggest that iron stringently represses *ptxR* expression under 20 %-SH, and in the late stationary phase (20–24 h) under 0 %-ST. This differs from the effect of iron on *toxA* expression (iron represses *toxA* expression under 20 %-SH and 0 %-ST) (Fig. 2).

### Level of DO within *P. aeruginosa* cultures

The above-described changes in the expression of *toxA* and *ptxR* may have been induced by variations in the level of DO within the *P. aeruginosa* cultures. Other investigators, using different methods to produce varying levels of EO when growing *P. aeruginosa*, reported that levels of DO within the culture medium were significantly reduced after 2–6 h incubation (Cooper *et al.*, 2003; Sabra *et al.*, 2002; Worlitzsch *et al.*, 2002). We used a DO meter to measure DO levels within the culture medium of PAO1 grown under 20 %-SH, 20 %-ST and 10 %-ST at each time point for each growth condition. (For cultures grown under 0 %-ST, anaerobic indicator strips confirmed that the DO level



**Fig. 7.** Level of DO in PAO1 cultures that were grown in shaking or static conditions under different levels of EO. Cells were prepared and grown in either iron-deficient or iron-sufficient medium for 22 h. The level of DO was determined at 2 h intervals using a DO meter. 20 %-SH, circles; 20 %-ST, triangles; 10 %-ST, squares; filled symbols, iron-deficient medium; open symbols, iron-sufficient medium. Values represent the means of three independent experiments.

within the medium reached 0% within 30 min of adding the Oxyrase For Broth.) Uninoculated TSB-DC medium was used to standardize the oxygen meter. In uninoculated TSB-DC incubated in parallel with the PAO1 cultures, the DO level remained at approximately 88% throughout the incubation time (data not shown). PAO1 cultures in iron-deficient medium had DO levels ranging from 60 to 75% at the 2 h time point (Fig. 7). By 6 h, the level of DO had dropped to 6–11%, regardless of growth condition (Fig. 7). For cultures grown in iron-sufficient medium, the levels also dropped from 78–88% (at the 2 h time point) to 5–15% (at 6 h) (Fig. 7). Similar to the levels in iron-deficient medium, the level of DO remained between 1 and 10% for all three conditions throughout the remainder of the growth cycle (Fig. 7). These results confirm studies by others, and indicate that regardless of the level of EO, or presence or absence of shaking, the growth of *P. aeruginosa* reduces the level of DO drastically (Kim *et al.*, 2003; Sabra *et al.*, 2002; Worlitzsch *et al.*, 2002).

## DISCUSSION

The goal of the present study was to determine if the expression of *toxA* and its regulatory gene *ptxR* changes when *P. aeruginosa* is grown in static cultures and under reduced levels of EO (two conditions that resemble the environment within the lung alveoli of CF patients). Our results indicate that both conditions contribute to increases in *toxA* and *ptxR* expression. The growth of PAO1 in a static liquid culture increased *toxA* expression, and changed the pattern of its expression, but did not interfere with the repression of *toxA* expression by iron, at least at later stages of growth (Fig. 2). Under the same conditions, *ptxR* expression was increased, but was deregulated with respect to iron (Fig. 6). These changes are not caused by differences in the levels of either EO (which is the same under both

20 %-SH and 20 %-ST) or DO within the PAO1 cultures. Under 20 %-SH and 20 %-ST, the level of DO after the 6 h time point, and throughout the remainder of the growth cycle, was 4–6 % (Fig. 7). In addition, no major difference in *toxA* or *ptxR* expression was detected between 20 %-ST and 10 %-ST, despite the difference in the level of EO (Fig. 2; data not shown).

Major phenotypic changes have been previously reported in *P. aeruginosa* strains grown in static vs shaking conditions. Deziel *et al.* (2001) reported that the growth of *P. aeruginosa* strain 57RP in a static liquid culture led to the spontaneous emergence of small-colony variants that were defective in swimming, swarming and twitching motilities. In addition, in comparison with their parent form (large-colony variants), the small-colony variants produced increased levels of pyocyanin and pyoverdine, but a reduced level of LasB. Wyckoff *et al.* (2002) also reported that the growth of the *P. aeruginosa* strain FRD1 in a static liquid culture gave rise to non-mucoid motile variants. This flagellum-mediated motility was associated with enhanced expression of the *fliC* gene (Wyckoff *et al.*, 2002). Switching the culture to shaking conditions reversed the enhancement in *fliC* expression, producing non-motile variants. Wyckoff *et al.* (2002) suggested that the phenomenon is related to the availability of oxygen. In static culture, the reduced level of oxygen forms a gradient that selects for motile variants able to swim to the highest level of oxygen at the meniscus (Wyckoff *et al.*, 2002). In shaking conditions, however, the equilibration of oxygen within the culture selected for stable non-motile variants instead (Wyckoff *et al.*, 2002). It is possible that the mechanism that produced changes in *toxA* and *ptxR* expression in PAO1 under 20 %-SH and 20 %-ST conditions is similar to the one that affects *fliC* expression. However, our results indicated that the level of DO in both 20 %-SH and 20 %-ST cultures is 4–6 % (Fig. 7). Therefore, the vigorous and continuous agitation of the culture under 20 %-SH may provide the cells with equal access to EO. In contrast, under 20 %-ST, cells are exposed to EO only at the meniscus, where they form a pellicle, as reported by Deziel *et al.* (2001) and Wyckoff *et al.* (2002). Thus, while the levels of EO and DO are similar under 20 %-SH and 20 %-ST, the limited access of the cells to EO in 20 %-ST may trigger the observed changes in *toxA* and *ptxR* expression. This scenario would explain the lack of major differences in *toxA* and *ptxR* expression under 20 %-ST and 10 %-ST, as cells have limited access to EO under both conditions.

In addition to the static condition, the elimination of oxygen from PAO1 cultures (0 %-ST) has an effect on *toxA* expression. The highest level in *toxA* expression occurred in PAO1 that was grown anaerobically (Fig. 2). The lack of this additional increase in *toxA* expression in PAO1 grown under 10 %-ST, despite the much reduced level of DO (4–6 %) (Fig. 7), indicates the necessity of the anaerobic environment to produce it. This increase was detected in cells regardless of the presence or absence of iron (Fig. 2). It is clear that the phenomenon is not caused by the presence

of the *toxA-lacZ* multi-copy plasmid pSW228. In addition, transcriptional analysis using real-time PCR revealed that the number of copies of *toxA* mRNA was significantly higher when PAO1, which carries a single chromosomal copy of *toxA*, was grown in 0 %-ST rather than 20 %-SH (data not shown). In addition, throughout the growth cycle, and in both iron-deficient and iron-sufficient media, the amount of ETA protein produced within the supernatant of PAO1 alone was considerably higher under 0 %-ST than under 20 %-SH (Fig. 3). Similar to *toxA* expression, *ptxR* expression reached its highest levels under 0 %-ST (Fig. 6). The increase in the expression of these two genes, despite the considerable decrease in PAO1 growth under 0 %-ST (Fig. 1), indicates that this phenomenon is not influenced by the change in the growth rate. At this time, our knowledge of *toxA* and *ptxR* regulation under anaerobic conditions is very limited. However, the significant increase in *toxA* and *ptxR* expression under 0 %-ST may represent part of the PAO1 adaptive response to anaerobic conditions. Under 0 %-ST, PAO1 may regulate *toxA* and *ptxR* expression through one of the anaerobic regulators, such as the anaerobic response regulator ANR (Zimmermann *et al.*, 1991). When *P. aeruginosa* is grown under 0 %-ST, ANR enhances the transcription of several genes, including those for the deaminase and denitrification pathways and for the extracellular virulence factor hydrogen cyanide (Galimand *et al.*, 1991; Sawyer, 1991; Zimmermann *et al.*, 1991). Under the same condition, ANR represses the expression of several genes of the *P. aeruginosa* aerobic respiratory pathway (Galimand *et al.*, 1991; Ray & Williams, 1997; Sawyer, 1991; Zimmermann *et al.*, 1991). ANR accomplishes this effect by recognizing a conserved sequence (TTGAC N<sub>x</sub> ATCAG) within the upstream regions of these genes (Winteler & Haas, 1996). To determine if ANR regulates the expression of *toxA* or *ptxR* directly, we searched the upstream regions of these genes for a potential ANR recognition site. While no ANR recognition site exists within the *toxA* upstream region, we detected a potential site (TTGAC N<sub>x</sub> ATCGG) that carries nine of the ten conserved residues within the *ptxR-ptxS* intergenic region (data not shown). However, the site is closer to the *ptxS* structural gene, located at 112 bp 5' end of the *ptxS* GTG start codon, than to *ptxR*. The significance of this site, and whether ANR plays a role (direct or indirect) in *toxA* and *ptxR* expression, are yet to be determined.

Based on available evidence, most of the enhancement in *toxA* expression seen under 20 %-SH in *P. aeruginosa* occurs through *pvdS* and *regA*. In addition, iron represses *toxA* expression in *P. aeruginosa* through these genes (Hamood *et al.*, 2004; Ochsner *et al.*, 1996). The biphasic pattern of *toxA* expression under these conditions is due to the differential expression of *regA* from two promoters, P1 and P2. The expression from P1 is iron insensitive, and occurs early in the growth cycle, while the expression from P2 is iron responsive, and occurs late in the growth cycle (Frank & Iglewski, 1988; Hamood *et al.*, 2004; Storey *et al.*, 1990; Wick *et al.*, 1990). Furthermore, other regulatory

genes may regulate *toxA* expression indirectly through either *regA* or *pvdS* (Hamood *et al.*, 1996b, 2004). However, evidence provided here suggests that, unlike those studies conducted under 20 %-SH, the increase in *toxA* expression that occurs under 0 %-EO does not occur through *regA* or *pvdS*. In contrast to the increase in *toxA* expression (Fig. 2), *pvdS* and *regA* expression was reduced (Fig. 4a, b). Although the enhancement in *toxA* expression under 0 %-ST may not occur through *pvdS* or *regA*, it may still require a functional PvdS or RegA, or both, i.e. it is not completely independent of these genes. As shown in Fig. 5, the loss of *pvdS* reduced the level of *toxA* expression by PAO1::*pvdS* in both 20 %-SH and 0 %-ST conditions. However, even in PAO1::*pvdS*, the increase in the level of *toxA* expression in 0 %-ST paralleled that detected in PAO1. This suggests that, although *pvdS* is required for maximum expression of *toxA*, the increase in *toxA* expression that is induced by anaerobic conditions is independent of *pvdS*.

Unlike *pvdS* and *regA* expression, and similar to *toxA* expression, *ptxR* expression in PAO1 was considerably increased under 0 %-ST (Fig. 6). In addition, under most conditions, the pattern of *ptxR* expression resembles that of *toxA* (Figs 2 and 6; data not shown). Although a logical conclusion is that the enhancement of *toxA* expression occurs through *ptxR*, it is less likely to be a possibility for several reasons. (1) Based on available findings, our current understanding is that *ptxR* enhances *toxA* expression through *regA*. In the presence of a *ptxR* plasmid, the expression of both *toxA* and *regA* was increased four- to fivefold (Hamood *et al.*, 1996b). Accordingly, if the increase in *toxA* expression at 0 %-ST occurs through *regA*, we would have detected an increase rather than a decrease in *regA* expression (Fig. 4a). (2) Under 0 %-ST, *toxA* expression increased, but was still repressed by iron, especially at the stationary phase of growth (Fig. 2), whereas *ptxR* expression was negatively regulated by iron under 20 %-SH only, and late in the growth cycle under 0 %-ST (Fig. 6). Therefore, if either static growth or EO regulates *toxA* expression through *ptxR*, *toxA* expression would have been deregulated with respect to iron.

A striking feature of *ptxR* expression in PAO1 was its deregulation with respect to iron under 20 %-ST, 10 %-ST and 0 %-ST (Fig. 6b; data not shown). Using RNase protection analysis, we have previously shown that *ptxR* is transcribed from two separate promoters (P1 and P2) in PAO1, producing T1 and T2 transcripts (Vasil *et al.*, 1998). Transcription from P1 is iron insensitive throughout the growth cycle, while that from P2 is iron regulated. Based on additional RNase protection experiments, we suggested that under microaerobic conditions (10 %-ST), iron negatively regulates P2 expression by Fur through *pvdS* (Vasil *et al.*, 1998). However, our present analysis showed that under 10 %-ST, and throughout the growth cycle of PAO1, *ptxR* expression did not differ between iron-sufficient and iron-deficient media (data not shown). In both studies, we utilized the GasPak Microaerobic Jar System to generate

the 10 %-ST conditions (Methods; Vasil *et al.*, 1998). However, while the RNase protection analysis measured the accumulation of T1 and T2 transcripts separately, the  $\beta$ -galactosidase assay that we used in this study measured the activity of both promoters. It is possible that under 20 %-ST, 10 %-ST and 0 %-ST, most of *ptxR* transcription is produced from the iron-insensitive P1 promoter. As a result, *ptxR* expression under these conditions is deregulated with respect to iron (Fig. 6b; data not shown). To examine this possibility, we have recently constructed a *ptxR*-*lacZ* fusion plasmid that carries the P2 promoter only. *ptxR* expression from this plasmid will be examined under different levels of EO, and compared with that produced by pJAC24.

Our results clearly show that the level of EO does not correlate with the level of DO within the PAO1 cultures. Regardless of the starting level of EO (20 or 10 %), and whether static or shaking, after 4–6 h of PAO1 culture growth, the level of DO was significantly reduced from as high as 88 % to ~6 % (Fig. 7). These data corroborate studies by others that utilized different methods to control the level of EO (Cooper *et al.*, 2003; Sabra *et al.*, 2002; Worlitzsch *et al.*, 2002). One possible reason for this phenomenon is the consumption of DO by the rapidly growing bacteria (Worlitzsch *et al.*, 2002). In addition, Sabra *et al.* (2002) suggested two mechanisms unique to *P. aeruginosa* that contribute to the sharp reduction in the level of DO: first, *P. aeruginosa* may block the transfer of EO from the gas phase into the liquid culture; and second, the polysaccharide capsule produced by *P. aeruginosa* may function as a physical barrier and interfere with the transfer of EO into the culture. In addition, Kim *et al.* (2003) recently proposed that limitation of iron in the growth medium interferes with transfer of oxygen into the PAO1 culture, significantly decreasing the partial pressure of oxygen (DO tension). They showed that, in iron-deficient medium only, the rate of oxygen transfer from the gas phase into the culture was significantly reduced (Kim *et al.*, 2003). However, our present analysis shows that in both iron-deficient and iron-sufficient media, and under all tested conditions, the level of DO was reduced considerably throughout the growth cycle of PAO1 (Fig. 7). This suggests that the sharp reduction in the level of DO is not produced by the limitation of iron in PAO1 culture. This apparent discrepancy is likely to be due to the different growth conditions utilized in each study. For example, while Kim *et al.* (2003) grew PAO1 in modified glucose medium in a bioreactor, and monitored the changes in a single shaking culture, we grew PAO1 in dialysed chelexed trypticase soy broth in individual flasks for each time point under the conditions described above, only one of which involved continuous shaking (20 %-SH). Another difference involved the level of iron in the iron-deficient medium. In this study, as in our previous studies, the level of iron in the iron-deficient medium was 0.05  $\mu\text{g ml}^{-1}$ , while Kim *et al.* (2003) reported the level of iron in their iron-deficient medium to be 0.6  $\mu\text{g ml}^{-1}$ . Other studies, however, in which PAO1 was grown in LB broth (which contains iron), also reported a

sharp reduction in the DO of the culture, providing indirect support for our results (Cooper *et al.*, 2003; Worlitzsch *et al.*, 2002).

One of the aims of this study was to examine the expression of *toxA* and *ptxR* under *in vitro* conditions that closely resemble *in vivo* conditions. Most *in vitro* growth conditions are designed to maximize the production of specific virulence factor(s). For example, the optimum *in vitro* growth conditions for ETA production by *P. aeruginosa* include iron-deficient medium (TSB-DC), 32 °C incubation and maximum aeration (aerobic, shaking at 250 r.p.m.) (Hamood *et al.*, 2004; Liu, 1973; Wick *et al.*, 1990). However, while iron deficiency and 32 °C may have their counterparts *in vivo* within the infected lungs of a CF patient, the equivalent of mixing by vigorous shaking is unlikely to occur. Rather, within the thick mucus that fills the alveoli, *P. aeruginosa* presumably grows under static conditions (Worlitzsch *et al.*, 2002) similar to the three static conditions described in this study. Worlitzsch *et al.* (2002) suggested that the severe hypoxic conditions within the thick mucus in CF airways are generated by two possible mechanisms. One mechanism is related to the severe reduction in mucus clearance in the CF airway, which increases oxygen consumption by the CF epithelium by two- to threefold (Worlitzsch *et al.*, 2002). The second mechanism involves the thickening of the stationary mucus due to the continuous mucus secretion (Worlitzsch *et al.*, 2002). As it penetrates the thick mucus, growth of *P. aeruginosa* would convert the hypoxic conditions to anaerobic. *P. aeruginosa* is considered to be an obligate aerobe, yet it grows under anaerobic conditions *in vitro* in the presence of nitrate as an electron acceptor (Hassett, 1996). The level of nitrate within the surface liquid of the CF airway is sufficient to sustain the growth of *P. aeruginosa* (Kim *et al.*, 2003; Linnane *et al.*, 1998). Thus, at least two of the conditions that we employed in our study (10 %-ST and 0 %-ST) appear to closely resemble the *in vivo* conditions within the CF airway. As shown in Fig. 2, we detected higher levels of *toxA* expression when we grew *P. aeruginosa* under static rather than shaking conditions. More importantly, the level of *toxA* expression increases further as the growth environment becomes anaerobic (0 %-ST) (Fig. 2). Based on these results, it is possible to assume that *P. aeruginosa* produces increased levels of ETA within the lungs of CF patients. Our results strongly support studies by others, which suggested that *toxA* is expressed in the *P. aeruginosa*-infected lungs of CF patients (Raivio *et al.*, 1994; Storey *et al.*, 1998).

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