INTRODUCTION

Bacterial communication (both inter- and intra-species) occurs through a well-developed system termed the quorum sensing (QS) system (Miller & Bassler, 2001; Whitehead et al., 2001). QS is a cell-density-dependent mechanism through which bacteria coordinate different activities including bioluminescence, plasmid conjugation and the production of different virulence factors (de Kievit & Iglewski, 2000; Rumbaugh et al., 2000). Intercellular signalling is accomplished through small N-acetylated homoserine lactone molecules termed autoinducers. Pseudomonas aeruginosa possesses at least two well-defined, interrelated QS systems, las and rhl, that control the production of different virulence factors, including elastases (LasB and LasA), alkaline protease, hydrogen cyanide, exotoxin A, pyocyanin, lectins, rhamnolipids and superoxide dismutase (de Kievit & Iglewski, 2000; Rumbaugh et al., 2000; Smith & Iglewski, 2003). Each QS system consists of two components, the autoinducer synthases (LasI and RhlI, respectively) and their cognate transcriptional regulators (LasR and RhlR, respectively) (de Kievit & Iglewski, 2000; Rumbaugh et al., 2000). LasI is the synthase for the autoinducer N-(3-oxododecanoyl) homoserine lactone (3OC12-HSL), while RhlI synthesizes the autoinducer N-butyryl homoserine lactone (C4-HSL) (de Kievit & Iglewski, 2000; Rumbaugh et al., 2000). At high cell density, 3OC12-HSL and C4-HSL reach critical levels and activate their regulators, which in turn enhance the transcription of different virulence genes (de Kievit & Iglewski, 2000; Rumbaugh et al., 2000).

Multiple studies have demonstrated the contribution of QS
to the pathogenesis of *P. aeruginosa*. Clinical studies suggested that QS systems are fully functional within infected tissues, especially the lungs of cystic fibrosis (CF) patients, who were chronically infected with *P. aeruginosa* (Erickson et al., 2002; Storey et al., 1992, 1997, 1998; Wu et al., 2000). Analysis of sputa from *P. aeruginosa*-infected CF patients revealed the presence of both *lasI* and *lasR* transcripts (Erickson et al., 2002; Storey et al., 1998). The accumulation of these transcripts correlated with the accumulation of transcripts for the QS-controlled genes *lasB*, *lasA* and *toxA* (Erickson et al., 2002; Storey et al., 1998). Additional studies confirmed the presence of either or both autoinducers within the sputum of CF patients (Geisenberger et al., 2000; Singh et al., 2000), while Erickson et al. (2002) showed that the autoinducers were biologically active.

The importance of QS in the virulence of *P. aeruginosa* has been demonstrated in different animal models of *P. aeruginosa* infections including the thermally injured mouse model and the mouse models of acute and chronic lung infections (Pearson et al., 2000; Rumbaugh et al., 1999a, c; Tang et al., 1996; Wu et al., 2001). These studies compared the virulence of *P. aeruginosa* mutants that carried deletions within QS genes with that of their parent strain. Rumbaugh et al. (1999a, c) showed that the mortality rate among thermally injured mice infected with QS mutants was significantly lower than that in mice infected with the parent strain. In addition, the mutants were significantly defective in their ability to spread either locally within the thermally injured skin or systemically within the bodies of the thermally injured/infected mice (Rumbaugh et al., 1999a, c). Using the mouse model of *P. aeruginosa* acute pulmonary infection, Pearson et al. (2000) showed that deletions within QS genes significantly reduced the lung damage caused by *P. aeruginosa*, as well as the mortality rate from the infection.

Since the QS systems control the production of different virulence factors, it is possible that the loss of one or both systems severely compromises the ability of *P. aeruginosa* to cause infections in humans. In this study, we tried to determine if QS-deficient strains of *P. aeruginosa* cause human infection, the rate at which they occur, and if their occurrence is associated with specific types of infections. We have identified and characterized five such QS-deficient isolates from a collection of 200 clinical isolates.

### METHODS

**Bacterial strains, media and growth conditions.** Bacterial strains used in this study are described in Table 1. *P. aeruginosa* strains were routinely grown in Luria–Bertani (LB) broth at 37°C (Ausubel et al., 1988). For LasB analysis, the strains were grown in LB broth at 37°C for 16 h with maximum aeration. For pyocyanin production, *P. aeruginosa* strains were grown in glycerol alanine minimal (GA) medium (1% v/v glycerol, 6 g L-alanine l⁻¹, 2 g MgSO₄ l⁻¹, 0·1 g K₂HPO₄ l⁻¹, 0·018 g

#### Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>P. aeruginosa</em> control strains</td>
<td></td>
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</tr>
<tr>
<td>PAO1</td>
<td>Prototroph; human isolate</td>
<td>Holloway et al. (1979)</td>
</tr>
<tr>
<td>PAOAlasB</td>
<td>ΔlasB derivative of PAO1, Gm⁺⁺</td>
<td>K. Rumbaugh, unpublished results</td>
</tr>
<tr>
<td>PDO100</td>
<td>rhlI::Tn501-2 derivative of PAO1, Hg⁺</td>
<td>Brint &amp; Ohman (1995)</td>
</tr>
<tr>
<td>PDO100/pJPP45</td>
<td>Carries rhlI under the lac promoter</td>
<td>Pearson et al. (1997)</td>
</tr>
<tr>
<td>PAO-JP2</td>
<td>Δ lasI derivative of PDO100, Hg⁺, Tc⁻</td>
<td>Pearson et al. (1997)</td>
</tr>
<tr>
<td>PAO-JP2/pECP61.5</td>
<td>Reporter strain for presence of autoinducer</td>
<td></td>
</tr>
<tr>
<td>PAO14AfgK</td>
<td>C₄-HSL; carries rhlA-lacZ plus lacp-rhlR, Cb⁺</td>
<td></td>
</tr>
<tr>
<td></td>
<td>fbgK::Tn5 derivative of PA14, Tc⁺</td>
<td>O'Toole &amp; Kolter (1998a)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> clinical isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CI-1</td>
<td>Wound infection (swab of wound site)</td>
<td>This study</td>
</tr>
<tr>
<td>CI-2</td>
<td>Lower respiratory infection (tracheal aspirate)</td>
<td>This study</td>
</tr>
<tr>
<td>CI-3</td>
<td>Lower respiratory infection (sputum)</td>
<td>This study</td>
</tr>
<tr>
<td>CI-4</td>
<td>Lower respiratory infection (tracheal aspirate)</td>
<td>This study</td>
</tr>
<tr>
<td>CI-5</td>
<td>Urinary tract infection (urine)</td>
<td>This study</td>
</tr>
<tr>
<td><em>A. tumefaciens</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTL4</td>
<td>Reporter strain for presence of autoinducer</td>
<td>C. Fuqua, personal communication</td>
</tr>
<tr>
<td></td>
<td>3OC₁₂-HSL; carries ptraB (pCF218) and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ptra1-lacZ (pCF372), Tc⁺ Smr⁺⁺</td>
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*Gm, gentamicin; †, resistant.
†Hg, mercury.
‡Tc, tetracycline.
§Cbr, carbenicillin.
¶Sm, streptomycin.
FeSO₄ 1⁻ at 37 °C for 24 h (MacDonald, 1963). For ExoT and ExoT production, cells were grown in deferrated dialysate of trypticase soy broth with 1% glycerol and 100 mM monosodium glutamate (TSBD-C) (Ohman et al., 1980) and containing nitritolactic acid as an inducing agent (TSBD-NTA) (Yahr et al., 1997). Agrobacterium tumefaciens NTL4 was grown overnight at 30 °C in minimal A medium (1X A) (10-5 g K₂HPO₄ l⁻¹, 4.5 g KH₂PO₄ l⁻¹, 1-0 g (NH₄)₂SO₄ l⁻¹, 0-5 g sodium citrate dihydrate l⁻¹; supplemented with 1-0 ml 1 M MgSO₄ 7H₂O and 10-0 ml 20% glycerol) for the 3OC₁₂-HSL cross-feeding bioassay (Miller, 1972). PTSB medium (5% peptone, 0-25% tryptic soy broth, pH 7-0) was utilized for the C₁₇-HSL cross-feeding bioassay (Pearson et al., 1997). Biofilm formation was examined using M63 minimal medium [13.6 g KH₂PO₄ l⁻¹, 2-0 g (NH₄)₂SO₄ l⁻¹, 0-5 mg FeSO₄ .7H₂O l⁻¹ (pH 7-0); supplemented with 0.2% (w/v) glucose, 1 mM MgSO₄ .7H₂O and 0.5% (w/v) Casamino acids] (Miller, 1972, O’Toole & Kolter, 1998b).

Clinical isolates. Two hundred P. aeruginosa isolates were obtained from specimens collected from patients at University Medical Center (UMC), Lubbock, TX. The isolates were identified biochemically (biotype analysis) as P. aeruginosa using the Microscan WalkAway Microbiology Analyser (Dade Behring) by the Clinical Microbiology Laboratory at UMC. Most of the isolates were obtained from patients with lower respiratory tract, urinary tract, or non-surgical or surgical wound infections; several of the isolates were obtained from spuTa of CF patients. The majority of the isolates represent one-time isolates from individual patients. However, a few isolates were obtained from different specimens from the same patient or were obtained from the same patient, same site over a period of time. Serotyping analysis (ERFA Canada) was done to determine if the isolates represented different strains based upon the O-antigen of the LPS. The isolates were streaked on LB agar and stored in 20 % glycerol/LB broth at -80 °C for 24–48 h. The presence of a clear zone around the growth streaks indicated LasA staphylolytic activity.

Pyocyanin assay. Pyocyanin was extracted from the supernatant fraction of P. aeruginosa isolates grown in GA medium for 24 h (Essar et al., 1990). A 5 ml sample of the supernatant was mixed with 5 ml chloroform and the lower organic layer was separated. To this layer, 1.5 ml 0-2 M HCl was added and the pyocyanin-rich organic layer was separated. The amount of pyocyanin within the extracted layer was determined by measuring the A₅₃₀.

Exotoxin A activity. Exotoxin A activity within the supernatant fraction of the isolates was determined using the ADP-ribose transferase assay as described previously (Vasil et al., 1977).

Cross-feeding bioassay for 3OC₁₂-HSL. This was done as described previously with some modifications (Fuqua & Winans, 1996; Fuqua et al., 1996; Stickler et al., 1998). The reporter strain A. tumefaciens NTL4, which is similar to the previously described strain A136 (Fuqua & Winans, 1996; Fuqua et al., 1996; Stickler et al., 1998), contains two plasmids: one carries the tral gene and the other carries the tral–lacz fusion (C. Fuqua, Indiana University, Bloomington, IN, personal communication). In the presence of 3OC₁₂-HSL, tral is activated and enhances the transcription of tral which leads to enhanced levels of β-galactosidase activity. Thus, the level of β-galactosidase activity produced by tral–lacz reflects the amount of autoinducers present in the tested supernatant samples. P. aeruginosa strains were grown in LB broth at 37 °C for 16 h. Cells were pelleted, and the supernatant fractions were separated and either immediately utilized in the assay or stored at -20 °C. NTL4 was grown overnight at 30 °C in LB broth. Samples of the overnight cultures were diluted in 1X A medium to an OD₆0₀ of 0-1. A 400 μl aliquot of each supernatant was added to 5 ml of the diluted NTL4 cultures and the cells were grown at 30 °C for 20–24 h. The cells were then pelleted, suspended in 200 μl distilled water and sonicated (VirSonic; The VirTis Company). The level of β-galactosidase activity within the lysate fraction was determined as described previously (Miller, 1972).

Cross-feeding bioassay for C₁₇-HSL. This was done using the PAO1 lasT/rhlB mutant PAO-JP2 carrying plasmid pECP61.5 (Pearson et al., 1997), which contains an rhlA–lacz fusion together with the rhlB gene expressed from the strong tac promoter (Pₜₐc). This plasmid serves as a reporter, for in the presence of exogenous C₁₇-HSL, RhlR is activated and enhances the expression of rhlA, which results in increased levels of β-galactosidase activity with very little background in the PAO-JP2 strain. Briefly, P. aeruginosa clinical isolates were grown in LB broth at 37 °C for 16 h. The supernatant fraction was isolated. The reporter strain PAO-JP2/pECP61.5 was grown overnight in LB broth at 37 °C. A 1 ml aliquot of this culture was pelleted, washed and resuspended in PTSB to an OD₆0₀ of 0-05. Cells were grown at 37 °C to an OD₆0₀ of 0-3 followed by the addition of 1 ml of the clinical isolates supernatant and growth for 1 h. Finally, cells were pelleted and the level of β-galactosidase activity was determined as described previously (Miller, 1972). PA01, PDO100 (rhl⁻) and the complemented PDO100/pJPP45 strains were used as controls. PDO100/pJPP45 contains multiple copies of the intact rhlB gene.

Immunoblotting experiments to detect LasB, ExoS and ExoT. The isolates were grown in either LB broth at 37 °C for 14 h for LasB production or in TSBD-NTA broth at 37 °C for 14 h for ExoT production. Proteins within the supernatant fraction were concentrated 10X with B15 Minicon Concentrators (Millipore). The amount of protein in each sample was determined by the method of Lowry et al. (1951). Equal amounts of protein from each sample were separated by 10% SDS-PAGE, (Hamood et al., 1996a; Laemmli, 1970), transferred to nitrocellulose membrane and probed with either polyclonal anti-LasB
or polyclonal anti-ExoS antibodies. The probed membranes were treated with anti-rabbit horseradish peroxidase-conjugated IgG and developed using SuperSignal West Pico chemiluminescent substrate (Pierce). Purified elastase (Elastin Products) was used as a positive control.

**PCR analysis of the QS genes.** Chromosomal DNA was extracted from PAO1 and the QS-deficient clinical isolates (Table 1) as described previously and utilized as templates in PCR experiments (Ausubel et al., 1988). Two different sets of oligonucleotide primers (one set for intact gene amplicons and the second for internal fragment amplicons) were designed by Primer Express (Applied Biosystems) corresponding to different regions within the lasI, lasR, rhlI and rhlR genes and synthesized (Integrated DNA Technologies) (Table 2). PCR was carried out in a total volume of 50 μl with 30 ng chromosomal DNA as a template. PCR conditions for the internal fragments included: heating at 95 °C for 5 min; followed by 32 cycles of 95 °C for 30 s, 59 °C for 60 s and 72 °C for 90 s; and a terminal cycle of 72 °C for 10 min. For the intact gene amplicons, PCR conditions for the amplification step were: 34 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min. Synthesized DNA fragments were detected on 1% agarose gels by ethidium bromide staining. Synthesis of the expected DNA fragments from the PAO1 template was confirmed by nucleotide sequence analysis (Biotechnology Core Facility, Texas Tech University).

**Analysis of biofilm initiation.** We examined the ability of the five *P. aeruginosa* QS-deficient clinical isolates to adhere to a polystyrene (abiotic) surface according to previously described protocols with some modifications (Deziel et al., 2001; O’Toole & Kolter, 1998a, b). Cells were grown overnight in LB broth and diluted in M63 medium to an OD600 of 0.02. One millilitre aliquots of the diluted cultures were dispensed in 12 × 75 mm polystyrene tubes and the tubes were incubated at 32 °C with gentle agitation for 10 h. The planktonic cells were decanted and their OD600 was determined. After thorough rinsing with distilled water, the biofilms in the tubes were stained by the addition of 1.0 ml 1% crystal violet. The tubes were then thoroughly washed with distilled water and the stain eluted from the biofilms by the addition of 4.0 ml 95% ethanol. The eluted crystal violet was measured at A590. PAO1 was used as a positive control and PA14ΔflgK was used as a negative control.

**Swimming motility.** The QS-deficient clinical isolates were grown overnight on LB agar plates at 37 °C. Individual colonies were then stabbed onto 1% tryptone/0.3% agar (w/v) swimming plates and the plates were incubated at 32 °C for 16 h (Deziel et al., 2001). Swimming motility was determined by measuring the diameter of the turbid zone around the inoculation site (Deziel et al., 2001).

**Twitching motility.** Individual colonies of the QS-deficient clinical isolates were stab-inoculated through the agar to the bottom of 1% LB agar plates (Deziel et al., 2001). The plates were incubated at 32 °C for 24 h. Bacterial growth at the interface between the plastic surface and the agar is indicative of twitching motility (Darzins, 1993; Deziel et al., 2001). To visualize the bacterial growth on the plastic surface, the agar was removed and the plate was stained with a 1% solution of crystal violet (Deziel et al., 2001). Twitching motility was determined by measuring the diameter of the stained growth (Deziel et al., 2001).

**Antibiotic susceptibility.** The broth microdilution method as outlined by the National Committee for Clinical Laboratory Standards (NCCLS, 2003) and performed using the Microscan WalkAway Microbiology Analyser was utilized to determine the minimum inhibitory concentration (MIC) of each antibiotic (UMC Clinical Microbiology Laboratory). The *P. aeruginosa* strain ATCC 27853 was used as an internal control.

**Statistical analysis.** The paired t-test (Statview; SAS) for significance was done to determine significant differences between control strains and the QS-deficient clinical isolates in all in vitro assays.

### RESULTS

#### Production of LasB by the *P. aeruginosa* clinical isolates

The main aim of this study was to determine if there are naturally occurring *P. aeruginosa* strains capable of causing a clinical infection despite the lack of functional QS. The existence of such a strain(s) would indicate that, while important, the QS systems are not absolutely essential for *P. aeruginosa* to establish infection, and that other QS-independent virulence factors can substitute for the loss of QS-controlled virulence factors/mechanisms. Two hundred isolates biochemically identified as *P. aeruginosa* were obtained from patients with lower respiratory tract, urinary tract, wound and other infections at University Medical Center, Lubbock, TX. All isolates were screened for LasB elastase deficiency. LasB production is stringently controlled by the las QS system; i.e. strains that carry a deletion in either the *las* or *lasR* gene are LasB deficient (de Kievit & Iglewski, 2000; Rumbaugh et al., 2000). Therefore, a LasB-deficient phenotype likely indicates a defect in one or both QS systems. The presence of elastolytic activity within the isolates was

### Table 2. The primers utilized in PCR experiments for amplicons of the QS genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>For intact QS gene amplicons</td>
<td>5’-ATGATCGTACAAATTTTGTCGCGC-3’</td>
<td>5’-GTCATGAAACGCCGACGTCG-3’</td>
</tr>
<tr>
<td>lasR</td>
<td>5’-ATGCGCGTTGAGCTGAGTTT-3’</td>
<td>5’-GAAAGATCAGAGACTGATACCC-3’</td>
</tr>
<tr>
<td>rhlI</td>
<td>5’-CTTGCGCTCATGCAATGCTGTC-3’</td>
<td>5’-AGGGGCTACGCTACACACAC-3’</td>
</tr>
<tr>
<td>rhlR</td>
<td>5’-CAATGGAGGAATGACGGAGG-3’</td>
<td>5’-GCTTCAATGAGGCCGACG-3’</td>
</tr>
</tbody>
</table>

| For internal QS gene amplicons | 5’-TCCGAGAGATGAAATCTGAGT-3’ | 5’-GCTCGATGCGCACTTCACG-3’ |
| lasR     | 5’-TGCGAGTTCTCGGGAGTACC-3’         | 5’-CGCGGAATAATTTCCCATATG-3’    |
| rhlI     | 5’-CGAATTGCTTCTGTAATGCTG-3’        | 5’-GGCTCATGCGGACGATGTA-3’      |
| rhlR     | 5’-TCAATAGCTACGCGCTATGGG-3’        | 5’-TCCAGAGGATGCGGCTCT-3’       |
examined by the elastin plate assay, which is specific and efficient, as several isolates can be screened on one plate (data not shown) (Schad et al., 1987). Five potential LasB-deficient clinical isolates (CI-1–CI-5) were identified (Table 1).

The LasB-deficient phenotype in these five isolates was confirmed by the elastin Congo red assay. As shown in Fig. 1(a), the levels of elastolytic activity produced by CI-1 through CI-5 were significantly \((P < 0.05)\) lower than that produced by PAO1 and similar to that produced by PAO\(\Delta\)lasB. To confirm these results, the presence of elastase protein within the supernatant of the isolates was examined by immunoblotting experiments using LasB-specific polyclonal antibodies. No LasB was detected within the supernatant fraction of strains CI-2, CI-3 and CI-4 (Fig. 1b). We excluded the possibility that these three isolates synthesized LasB but were defective in its secretion to the extracellular environment by additional immunoblotting experiments on the lysate fractions. Neither LasB nor LasB cross-reactive materials were detected (data not shown). A faintly reactive band that corresponds to the 33 kDa mature LasB was detected within the supernatant of CI-5 (Fig. 1b). The presence of this faint band suggests that LasB is produced and initially processed, but at a considerably reduced level. Isolate CI-1 produced a smaller cross-reactive product (Fig. 1b). Whether this band represents a degradation product of the mature LasB protein or an abnormally processed form of LasB is not known at this time.

Production of autoinducers by the LasB-deficient isolates

Next, we screened the five LasB-deficient clinical isolates for the presence of a functional las QS system by determining the level of autoinducer 3OC\(_{12}\)-HSL produced by the isolates using the 3OC\(_{12}\)-HSL cross-feeding bioassay as described in Methods. This assay is based on the fact that interspecies communication occurs among Gram-negative bacteria via their autoinducers. The positive control strain, PAO1, usually produces a considerable amount of the autoinducers while the negative control strain, PAO-JP2, which carries a mutation in both QS systems, produces none (Fig. 2a). As shown in Fig. 2(a), the amount of 3OC\(_{12}\)-HSL produced by the five CIs was significantly \((P < 0.05)\) lower than that produced by PAO1, with CI-5 producing the highest level of autoinducers and CI-2 the lowest level (Fig. 2a).

**Fig. 1.** Examination of the five potential LasB-deficient CIs for LasB activity and synthesis. (a) Elastin Congo red assay for levels of elastolytic activity \((A_\text{elas})\) within the supernatant fraction of the isolates. Cells were grown in LB broth at 37 °C for 16 h. The cultures were adjusted to an \(OD_{540}\) of 3.5–4.0 before harvesting to eliminate growth-related variations in elastolytic activity. PAO1 was used as a positive control and the LasB-deficient mutant PAO\(\Delta\)lasB as a negative control. Values represent the mean of three independent experiments ± SD. (b) Detection of LasB protein within the supernatant fraction of the five isolates. Cells were grown and harvested as above. Proteins within the supernatant fraction were concentrated 10× and 100 µg protein from each sample were separated by SDS-PAGE. LasB was detected by immunoblotting with elastase-specific antibody. Lanes: (1) purified LasB; (2) PAO1; (3) PAO\(\Delta\)lasB; (4–8) CI-1–5.

**Fig. 2.** Levels of 3OC\(_{12}\)-HSL and C\(_{4}\)-HSL autoinducers within the supernatant fractions of the LasB-deficient clinical isolates. Cells were grown in LB broth at 37 °C for 16 h. The cultures were adjusted to an \(OD_{540}\) of 3.5–4.0 before harvesting. The level of autoinducer was determined as Miller units of \(\beta\)-galactosidase activity in the cross-feeding bioassay as described in Methods. PAO1 served as the positive control while its isogenic QS-deficient mutants PAO-JP2 and PDO100 (Table 1) were used as negative controls. (a) Levels of 3OC\(_{12}\)-HSL present in supernatants of controls and CIs. (b) Levels of C\(_{4}\)-HSL present in supernatants of controls and CIs. The rhl\(_{\text{def}}\) defect of PDO100 was complemented by pJPP45, which carries intact rhl. Values represent the mean of three independent experiments ± SD.
Using a second cross-feeding bioassay (Pearson et al., 1997) we tried to determine if one or more of the elastase-deficient isolates produced the autoinducer C4-HSL. As shown in Fig. 2(b), PAO1 produced a considerable amount of C4-HSL while the rhlI- mutant PDO100 (negative control) produced very little; this amount was subtracted as background. We detected similar levels of C4-HSL in the complemented mutant PDO100/pPP45 versus PAO1. Similar to PDO100, CI-1, -2, -3 and -4 produced no C4-HSL (Fig. 2b). However, CI-5 produced detectable levels of C4-HSL (Fig. 2b). These results suggest that CI-1, -2, -3 and -4 are defective in the production of both autoinducers (3OC12-HSL and C4-HSL) while CI-5 is not completely defective in the production of either autoinducer.

Examining the QS-deficient isolates for the presence of QS-genes

From this point forward, the five CIs with reduced or absent autoinducer activity will be referred to as QS-deficient. The failure of the CIs to produce autoinducers may be due to the loss of any one of the genes that code for different components of the QS systems. We examined this possibility by attempting to synthesize DNA fragments carrying intact rhlI, lasI, lasR and rhlR or internal regions within each gene. Oligonucleotide primers used for the different PCR experiments are shown in Table 2. PAO1, which carries intact QS genes, was used as a positive control. As shown in Fig. 3(a) and (e), a 625 bp fragment that carries intact rhlI as well as a 143 bp internal rhlI fragment were synthesized from the chromosome of PAO1 and all five isolates. With respect to lasI, a 605 bp fragment that carries intact lasI was synthesized from the chromosome of PAO1 and CI-1, -2, -3 and -5 (Fig. 3b) but not CI-4; whereas a 363 bp internal lasI fragment was synthesized from PAO1 and all five isolates (Fig. 3f). A 725 bp fragment that carries intact lasR as well as a 362 bp internal lasR fragment were synthesized from the chromosome of PAO1 and all five isolates (Fig. 3f). For rhlR, a 730 bp fragment that carries intact rhlR was synthesized from PAO1, CI-1 and -5 but not CI-2, -3 and -4 (Fig. 3d). However, a 207 bp internal rhlR fragment was synthesized from PAO1, CI-1, -3 and -5 but not CI-2 and -4 (Fig. 3h).

These results strongly suggest that CI-2 and CI-4 lack the lasR and rhlR genes. In addition, while we failed to synthesize DNA fragments that carry intact rhlR and lasI from CI-3 and CI-4, respectively (Fig. 3b and d), we synthesized internal rhlR and lasI fragments from both strains (Fig. 3f and h). The most likely explanation for these discrepancies is that CI-3 and CI-4 carry changes within the regions that flank the rhlR and lasI genes, respectively. These changes would interfere with the hybridization of the primers and prevent the synthesis of the PCR products. In contrast, the absence of changes within the genes allowed the synthesis of the internal fragments. Currently, we are trying to clone larger DNA fragments from CI-3 and CI-4 that carry rhlR and lasI, respectively. The intact gene will then be isolated from each fragment and its nucleotide sequence will be determined.

Production of pyocyanin, LasA and exotoxin A by the QS-deficient clinical isolates

To further analyse the QS-deficient phenotype of the five CIs, we examined them for the production of two other stringently QS-controlled factors, LasA and pyocyanin. The production of LasA is controlled by the las system while pyocyanin production is directly regulated by the rhl system (de Kievit & Iglewski, 2000; Rumbaugh et al., 2000). However, due to the hierarchical nature of the QS systems and the importance of the las system in the hierarchy, pyocyanin production should also be regulated by the las system (de Kievit & Iglewski, 2000; Rumbaugh et al., 2000). We utilized GA medium to determine the production of pyocyanin by the QS-deficient clinical isolates (MacDonald, 1963). Analysis of the QS-deficient CIs revealed that, similar to PDO100, CI-4 produced no pyocyanin whereas CI-1, CI-2 and CI-3 produced negligible levels of pyocyanin in comparison with PAO1 (Fig. 4). In contrast, the level of pyocyanin produced by CI-5 was comparable to that produced by PAO1 (Fig. 4).

The production of the LasA-associated staphylolytic activity by the QS-deficient CIs was examined as described in Methods. As shown in Table 3, CI-5 produced limited staphylolytic activity while the other QS-deficient CIs produced no staphylolytic activity. These results strongly support the possibility that these isolates carry some type of defect in their QS systems.

![Fig. 3. Ethidium bromide-stained 1% agarose gels showing the amplified intact genes (a–d) and internal fragments (e–h) of (a & e) rhlI, (b & f) lasI, (c & g) lasR and (d & h) rhlR. The fragments were amplified from the chromosomes of PAO1 and the QS-deficient clinical isolates by PCR using the primers described in Table 2. Lanes: (1) 100 bp molecular size standard; (2) no template control; (3) PAO1; (4–8) CI-1 through CI-5. Arrowheads indicate amplicons for each gene. Sizes of relevant molecular size standards and the amplicons are given in bp.](image-url)
We also examined the level of exotoxin A activity within the supernatant fractions of the CIs. Exotoxin A, which is not stringently controlled by the QS system, is an ADP-ribosyl transferase enzyme that modifies elongation factor-2 within the eukaryotic cell resulting in cell death. As shown in Table 3, exotoxin A activity was detected at variable levels within all the clinical isolates. CI-1 and CI-3 produced only 26–27% of exotoxin A activity of PAO1, while CI-5 produced similar activity (123%), and CI-2 (165%) and CI-4 (288%) produced considerably more activity.

Production of type III secretion system (TTSS) proteins ExoS and ExoT by the QS-deficient isolates

The recently described \textit{P. aeruginosa} virulence-associated TTSS consists of many components including the effector proteins ExoS, ExoT, ExoU and ExoY (Finck-Barbancon \textit{et al.}, 1997; Ganesan \textit{et al.}, 1998; Krall \textit{et al.}, 2000; Yahr \textit{et al.}, 1998). These effector proteins are directly injected from the cytoplasm of \textit{P. aeruginosa} into the eukaryotic cell upon contact. Several previous studies confirmed the importance of the effector proteins in the pathogenesis of \textit{P. aeruginosa} (Feltman \textit{et al.}, 2001; Finck-Barbancon \textit{et al.}, 1997; Hauser & Engel, 1999; Henriksson \textit{et al.}, 2000; Olson \textit{et al.}, 1999; Roy-Burman \textit{et al.}, 2001). As the ADP-ribosylating proteins ExoS and ExoT are highly homologous (ExoS antibody cross-reacts with ExoT; Yahr \textit{et al.}, 1996), we examined the presence of ExoS and ExoT within the supernatant fractions of the isolates by immunoblotting experiments using ExoS polyclonal antibody. As shown in Fig. 5, only CI-3 produced ExoS and ExoT. CI-2 and CI-4 produced ExoT only while CI-1 and CI-5 produced neither. At this time we do not know whether any of the five CIs produce either ExoU or ExoY.

Initiation of biofilm formation by the QS-deficient clinical isolates

Bacterial biofilms are highly structured communities attached to biotic or abiotic surfaces and surrounded by a

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Pyocyanin production by the QS-deficient clinical isolates. Cells were grown in GA medium at 37 °C for 24 h, the supernatant fractions were separated, and the amount of pyocyanin (µg ml\textsuperscript{-1}) in each fraction was determined by the chloroform:acid extraction procedure (Methods). PAO1 and PDO100 (rhl\textsuperscript{+/-}) served as positive and negative controls, respectively. Values represent the mean of three independent experiments ± SD.}
\end{figure}

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Strain & \text{LasB} & \text{LasA} & \text{Exotoxin A} & \text{3OC12-HSL} & \text{C4-HSL} & \text{Pyocyanin}\text{(µg ml\textsuperscript{-1})}\text{*} & \text{Biofilm} \text{initiation}\text{†} & \text{Swimming} & \text{Twitching} \\
\hline
PAO1 & 100 & 100 & 100 & 100 & 100 & 100 & + & 100 & 100 \\
CI-1 & 6 & 26 & 3 & 1 & 0 & 0 & + & 1 & 1 \\
CI-2 & 13 & 165 & 2 & 2 & 0 & 0 & + & 1 & 1 \\
CI-3 & 13 & 27 & 2 & 2 & 0 & 0 & + & 1 & 1 \\
CI-4 & 13 & 91 & 2 & 2 & 0 & 0 & + & 1 & 1 \\
CI-5 & 6 & 123 & 2 & 91 & 91 & 91 & + & 1 & 1 \\
\hline
\textbf{Table 3. Summary of the different phenotypes of the QS-deficient \textit{P. aeruginosa} clinical isolates} & \textbf{Strain} & \textbf{LasB} & \textbf{LasA} & \textbf{Exotoxin A} & \textbf{3OC12-HSL} & \textbf{C4-HSL} & \textbf{Pyocyanin} & \textbf{Biofilm} \text{initiation} & \textbf{Swimming} & \textbf{Twitching} \\
\hline
\textit{P. aeruginosa} & 100 & 100 & 100 & 100 & 100 & 100 & + & 100 & 100 \\
CI-1 & 6 & 26 & 3 & 1 & 0 & 0 & + & 1 & 1 \\
CI-2 & 13 & 165 & 2 & 2 & 0 & 0 & + & 1 & 1 \\
CI-3 & 13 & 27 & 2 & 2 & 0 & 0 & + & 1 & 1 \\
CI-4 & 13 & 91 & 2 & 2 & 0 & 0 & + & 1 & 1 \\
CI-5 & 6 & 123 & 2 & 91 & 91 & 91 & + & 1 & 1 \\
\hline
\end{tabular}
\caption{Summary of the different phenotypes of the QS-deficient \textit{P. aeruginosa} clinical isolates}
\end{table}
glycocalyx. Within the infected host, bacterial biofilms are resistant to host defences and antibiotic treatment. *P. aeruginosa* forms biofilms on different infected tissues including the lungs of CF patients. Biofilm development involves specific stages: initiation, maturation and detachment (Costerton et al., 1999). The *P. aeruginosa* QS systems appear to be involved at all three stages (Davies et al., 1998; de Kievit et al., 2001). Therefore, we tried to determine if the lack of functional QS affected the ability of the CIs to initiate biofilms on polystyrene (abiotic) surfaces as described in Methods. PAO1, which initiated a strong biofilm, was used as the 100% standard to compare the ability of QS-deficient CIs to initiate biofilm (Fig. 6). The isolates were also compared to the flagellum mutant PA14ΔflgK, which forms a defective biofilm. As shown in Fig. 6, with the exception of CI-5, the QS-deficient isolates initiated biofilms significantly (P < 0.05) less efficiently than PAO1 (29–37%). CI-5 initiated biofilm at 82% of the capacity of PAO1 (Fig. 6). In comparison with PA14ΔflgK (21% of the capacity of PAO1), all of the clinical isolates initiated biofilms more efficiently (Fig. 6). These results suggest that their QS defects might have affected the ability of the isolates to initiate biofilm formation. However, the possible QS defect in CI-5 only slightly reduced its ability to produce pyocyanin and to initiate biofilm (Figs 4 and 6), suggesting its *rhl* QS system is intact.

**Examining the QS-deficient clinical isolates for swimming and twitching motilities**

In addition to QS, the initiation of biofilm formation by *P. aeruginosa* depends on two cell-associated structures; the flagellum and type IV pili (O’Toole & Kolter, 1998a, b). Both types of motility are important in the initial stages of biofilm formation by *P. aeruginosa* (O’Toole & Kolter, 1998a, b). Therefore, we tried to determine if any of the QS-deficient isolates was defective in either one or both motilities. On swimming plates, the motile strain PAO1 was used as the 100% standard for motility while the non-motile strain PA14ΔflgK was used as a negative control. The isolates produced swimming zones ranging from 24 to 100% (Table 3) compared to PAO1. For example, CI-1, CI-3 and CI-5 produced swimming zones less than 50% of those of PAO1 (Table 3) while CI-2 and CI-4 produced zones comparable to that produced by PAO1 (92 and 100%) (Table 3). With respect to twitching motility, CI-2, CI-4 and CI-5 produced zones of twitching motility 92–100% of that produced by PAO1 (Table 3). CI-1 and CI-3 produced zones only 31 and 15% of the size of PAO1, respectively (Table 3). These results suggest that none of the isolates is non-flagellated or carry a non-motile flagellum. In addition, none of the isolates appear to be completely lacking in twitching motility. Further analysis, such as immunoblot experiments using specific flagellum and type IV pili antibodies, would be required to confirm these results.

**Antibiotic susceptibility profiles of the QS-deficient isolates**

One of the virulence attributes of *P. aeruginosa* is its resistance to different antibiotics. This resistance is provided by several multi-drug resistance (MDR) pumps. It has been shown that the resistance of a specific *P. aeruginosa* strain to antibiotics varies depending on the number of MDR pumps expressed. We examined the resistance of the isolates to four types of antibiotics: anti-pseudomonal penicillins, cephalosporins, fluoroquinolones and aminoglycosides. As shown in Table 4, the isolates varied in their resistance to these antibiotics. Notably, CI-5 was susceptible only to imipenem (Table 4).

**Correlating the QS-deficient phenotype with type of infection**

The two hundred clinical isolates were obtained from a variety of infections including lower respiratory tract, urinary tract and wound infections. As shown in Table 1,
Table 4. Antibiotic susceptibilities of the clinical isolates

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>ATCC 27853*</th>
<th>PAO1</th>
<th>CI-1</th>
<th>CI-2</th>
<th>CI-3</th>
<th>CI-4</th>
<th>CI-5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antipseudomonal penicillins/carbapenem/monobactams</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipercillin</td>
<td>$\leq 8$, S†</td>
<td>$\leq 8$, S</td>
<td>$32$, S</td>
<td>$\leq 8$, S</td>
<td>$\leq 8$, S</td>
<td>$\leq 8$, S</td>
<td>$&gt;64$, R</td>
</tr>
<tr>
<td>Pipericillin/tazobactam</td>
<td>$\leq 8$, S</td>
<td>$\leq 8$, S</td>
<td>$32$, S</td>
<td>$\leq 8$, S</td>
<td>$\leq 8$, S</td>
<td>$\leq 8$, S</td>
<td>$&gt;64$, R</td>
</tr>
<tr>
<td>Imipenem</td>
<td>$\leq 4$, S</td>
<td>$&gt;8$, R</td>
<td>$\leq 4$, S</td>
<td>$\leq 4$, S</td>
<td>$&gt;8$, R</td>
<td>$\leq 4$, S</td>
<td>$\leq 4$, S</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>$\leq 8$, S</td>
<td>$\leq 8$, S</td>
<td>$\leq 8$, S</td>
<td>$\leq 8$, S</td>
<td>$\leq 8$, S</td>
<td>$\leq 8$, S</td>
<td>$&gt;16$, R</td>
</tr>
<tr>
<td><strong>3rd and 4th generation cephalosporins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefepime</td>
<td>$\leq 2$, S</td>
<td>$\leq 2$, S</td>
<td>$4$, S</td>
<td>$8$, S</td>
<td>$16$, I</td>
<td>$8$, S</td>
<td>$&gt;16$, R</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>$\leq 8$, S</td>
<td>$\leq 8$, S</td>
<td>$\leq 8$, S</td>
<td>$\leq 8$, S</td>
<td>$16$, I</td>
<td>$&gt;16$, R</td>
<td></td>
</tr>
<tr>
<td>Fluoroquinolone</td>
<td>$\leq 1$, S</td>
<td>$\leq 1$, S</td>
<td>$&gt;2$, R</td>
<td>$\leq 1$, S</td>
<td>$\leq 1$, S</td>
<td>$\leq 1$, S</td>
<td>$&gt;2$, R</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>$8$, S</td>
<td>$8$, S</td>
<td>$\leq 2$, S</td>
<td>$16$, S</td>
<td>$16$, S</td>
<td>$32$, I</td>
<td>$&gt;32$, R</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>$2$, S</td>
<td>$4$, S</td>
<td>$\leq 1$, S</td>
<td>$&gt;8$, R</td>
<td>$8$, I</td>
<td>$&gt;8$, R</td>
<td>$&gt;8$, R</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>$\leq 1$, S</td>
<td>$\leq 1$, S</td>
<td>$\leq 1$, S</td>
<td>$2$, S</td>
<td>$2$, S</td>
<td>$4$, S</td>
<td>$&gt;8$, R</td>
</tr>
</tbody>
</table>

*Reference strain used by UMC Clinical Laboratory for quality control of MIC.
†S, susceptible; R, resistant; I, intermediate.

the QS-deficient isolates were clustered to lower respiratory tract infections (3/5); CI-2, CI-3 and CI-4 were isolated from tracheal aspirates or sputum. Isolate CI-1 was obtained from a wound and CI-5 was obtained from urine.

CI-1 was obtained as a pure culture from an infected wound, however, no further data were available on this patient. CI-2 and CI-4 were isolated from tracheal aspirates obtained 1 month apart from a 50-year-old male admitted with a primary diagnosis of a benign pituitary tumour. The isolates produced the same biotype and both were serotype 11. In addition, the isolates have similar phenotypic characteristics including: the production of virulence factors, biofilm initiation and motility (Figs 1, 4, 5, 6 and Table 3). This indicated that CI-2 and CI-4 represent a single *P. aeruginosa* strain that was isolated twice from the same patient. The isolates differ only in their resistance to certain antibiotics (CI-4 is intermediate rather than susceptible to ceftazidime and amikacin; Table 4) and in changes in our ability to recover intact lasI from CI-4 (Fig. 3d). At this time, it is not known whether these variations occurred in response to antibiotic treatment or other in vivo conditions. Clinical data indicated that CI-2 represented colonization while CI-4 had produced infection. CI-3 was obtained from an 86-year-old male whose primary diagnosis was chronic obstructive pulmonary disease/chronic *P. aeruginosa* infection. The strain was obtained as a pure culture from sputum and clinical data showed the isolate met the criteria for lower respiratory infection. CI-5 was obtained from an 82-year-old male admitted to the hospital with sepsis secondary to urinary tract infection (indwelling catheter). The strain was isolated as a pure culture from catheterized urine; clinical data revealed this isolate to be the aetiologic agent of the patient’s infection.

These results suggest that lack of functional QS does not interfere with the ability of *P. aeruginosa* to cause lower respiratory tract, urinary tract or wound infections.

**DISCUSSION**

*P. aeruginosa*, which causes many different types of infections in humans, is multi-factorial in its virulence. Depending on the infected tissue, some virulence factors play more important roles in the pathogenesis of *P. aeruginosa* infections than others. The contribution of individual factors to *P. aeruginosa* virulence in natural infections has been examined by several clinical studies (Feltman *et al.*, 2001; Fleiszig *et al.*, 1996; Hamood *et al.*, 1996b; Hirakata *et al.*, 2000; Roy-Burman *et al.*, 2001; Rumbaugh *et al.*, 1999b). These studies showed that during infections other virulence factors appear to compensate for the loss of any single virulence factor (Feltman *et al.*, 2001; Fleiszig *et al.*, 1996; Hamood *et al.*, 1996b; Hirakata *et al.*, 2000; Rumbaugh *et al.*, 1999b). Strains defective in the production of exotoxin A, LasB, exoenzyme S, ExoU or other factors individually caused infections of the ear, lower respiratory tract, urinary tract and wounds (Feltman *et al.*, 2001; Fleiszig *et al.*, 1996; Hamood *et al.*, 1996b; Hirakata *et al.*, 2000; Rumbaugh *et al.*, 1999b).

What is not known is the impact of simultaneous loss of several virulence factors on the ability of *P. aeruginosa* to...
isolates produced reduced levels of LasB, 3OC12-HSL, which produced a nearly wild-type level of pyocyanin, the virulence factor from three types of infections; lower respiratory tract (3), urinary tract (1) and wound (1). With the exception of CI-5, which produced a nearly wild-type level of pyocyanin, the isolates produced reduced levels of LasB, 3OC12-HSL, pyocyanin and LasA (Figs 1, 2, 4 and Table 3). In addition, isolates CI-2 and CI-4 (which are the same strain isolated first as a colonizer and 1 month later from lower respiratory tract infection) carry neither lasR nor rhlR genes (Fig. 3b and d) and CI-4 appears to have sustained a mutation in its lasI gene (Fig. 3b). Furthermore, most of the isolates initiated biofilm poorly (Fig. 6). These results suggest that \textit{P. aeruginosa} is capable of causing different infections despite the loss of optimum QS.

The approach we utilized in the present study was the opposite to that taken in studies reported previously. Those studies, which showed the important role played by the QS systems in \textit{P. aeruginosa} pathogenesis, examined the presence of \textit{P. aeruginosa} autoinducers as well as transcripts of the QS and QS-controlled genes within clinical samples, including sputa, from CF patients (Erickson et al., 2002; Geisenberger et al., 2000; Pearson et al., 2000; Rumbaugh et al., 1999a, c; Singh et al., 2000; Storey et al., 1992, 1997, 1998; Wu et al., 2000). However, we obtained five QS-defective CIs from three types of infections; lower respiratory tract (3), urinary tract (1) and wound (1). With the exception of CI-5, which produced a nearly wild-type level of pyocyanin, the isolates produced reduced levels of LasB, 3OC12-HSL, pyocyanin and LasA (Figs 1, 2, 4 and Table 3). In addition, isolates CI-2 and CI-4 (which are the same strain isolated first as a colonizer and 1 month later from lower respiratory tract infection) carry neither lasR nor rhlR genes (Fig. 3b and d) and CI-4 appears to have sustained a mutation in its lasI gene (Fig. 3b). Furthermore, most of the isolates initiated biofilm poorly (Fig. 6). These results suggest that \textit{P. aeruginosa} is capable of causing different infections despite the loss of optimum QS.

The strongest evidence for the involvement of the QS systems in the virulence of \textit{P. aeruginosa} comes from animal models such as the thermally injured mouse model, the acute pneumonia model and the chronic lung infection model (Pearson et al., 2000; Rumbaugh et al., 1999a, c; Tang et al., 1996). For example, strain PAO-JP2, which carries deletions within lasI and rhlR, was ~15-fold less virulent than its parent strain PAO1 in the thermally injured mouse model (Rumbaugh et al., 1999c). However, experiments involving animal models are conducted under controlled laboratory conditions. Therefore, in a natural infection, PAO-JP2 may not be attenuated as much in its virulence despite the loss of QS. The finding that CI-2 and CI-4 carry defective lasR and rhlR genes (Fig. 3b and d) supports this possibility. Comparative analysis with the similar isogenic strain PAO-JP3 in the lung model or the thermally injured mouse model may help us determine whether their virulence is compromised.

Results of this study demonstrate the complicated process that governs the production of \textit{P. aeruginosa} virulence factors during different infections. CI-1, CI-2, CI-3 and CI-4 clearly do not produce the QS stringently controlled factors LasB, pyocyanin and LasA (Figs 1, 4b and Table 3). Since the CIs produced infections, the virulence of these isolates may, therefore, be due to the enhanced production of either factors that are less stringently controlled by QS (or can be regulated in other ways besides QS) or factors yet uncharacterized that are induced \textit{in vitro}. As examples of the first possibility, CI-2 and CI-4 produced high levels of exotoxin A (Table 3) while CI-3 produced more ExoT and ExoS than PAO1 (Fig. 5). On the other hand, analysis of CI-1 clearly supports the latter possibility. This isolate lacked all the virulence factors tested, yet it still caused a wound infection.

At this time, the mechanism(s) that led to the QS-deficient phenotype in these isolates is not known. It is clear that it is not an \textit{in vivo}-induced phenomenon. The assays for the production of autoinducers and QS-controlled factors were conducted \textit{in vitro}, eliminating any \textit{in vivo} repression, yet the levels remained low. We also excluded the possibility that the production of low levels of autoinducers occurs in all \textit{P. aeruginosa} CIs by examining the levels produced by several of the elastase-positive isolates. All produced levels of autoinducers comparable to that of PAO1 (data not shown). Except for CI-2 and CI-4, which carry simultaneous deletions in the lasR and rhlR genes, the other CIs carry all four QS genes (Fig. 4). The fact that an internal amplicon was detected in CI-3 but not the intact gene of rhlR and a similar situation for lasI in CI-4, indicates that there may be enough base pair mismatches in the region(s) of the primer(s) to prevent amplification of a gene product. It is possible that these CIs carry point mutations within one or more of the QS.
structural genes rendering them non-functional. Another possibility is that the CIs carry point mutations within the upstream regions of the QS genes that would interfere with the transcription of these genes. We are currently examining this possibility through complementation analysis using plasmids carrying individual QS genes. Alternatively, the QS-deficient CIs could carry mutations within one of the global regulatory genes such as gacA or vfr that regulate expression of all QS genes (Albus et al., 1997; Reimmann et al., 1997).

It is also possible that rearrangement of the QS genes occurred in vivo. Such rearrangements may alter the production of different virulence factors even after the strain is isolated and analysed in vitro. Rearrangements within the upstream region of the toxA gene from different clinical isolates have been previously reported (Pritchard & Vasil, 1990; Sokol et al., 1994). In addition, a recent study indicated that certain P. aeruginosa strains are hypermutable (Oliver et al., 2000), i.e. they have a higher mutation rate than the wild-type (Miller, 1996). Our current analysis of CI-2 and CI-4 revealed that the persistence of the P. aeruginosa strain within the respiratory tract for 30 days had no major impact on its virulence phenotype (Figs 1, 2, 4, 5, 6 and Table 3). However, this persistence appeared to influence the susceptibility of the strain to certain antibiotics (Table 4) and may have led to a mutation within the las gene (Fig. 3b). As an extension of the present study, we plan to analyse by PCR and nucleotide sequence analysis multiple isolates of the same P. aeruginosa strain obtained from a single chronically infected patient over an extended period of time for possible rearrangements within the QS or QS-controlled genes.

There are several additional variables that may influence the ability of a QS-deficient strain to cause infection in vivo: antibiotic treatment, the presence of multiple P. aeruginosa strains and the presence of other bacteria in the infection. We have previously shown that in burn patients the length of antibiotic treatment influences the production of several virulence factors by P. aeruginosa (Griswold et al., 2001). Except for CI-5, the CIs were not exceptionally resistant to antibiotics (Table 4). A single patient may be infected by multiple strains of P. aeruginosa, each producing different levels of autoinducers and/or QS-controlled factors. Certainly mixed infections with two or more genera of bacteria that support each other are well-known clinical problems. Either case may assist a QS-deficient strain to participate in an infection.

CI-5 appears to have a unique phenotype with respect to pyocyanin production and biofilm initiation. Analysis of CI-5 suggested that it carries functional yet inefficient las and rhl systems. The amount of 3OC12-HSL and C12-HSL produced by CI-5 were significantly lower than those produced by PAO1 (20 and 34%, respectively; Table 3). CI-5 also produced intact LasB but at a drastically reduced level than PAO1 (Fig. 1b). However, the level of pyocyanin produced by CI-5 was significantly higher than those produced by the other QS-deficient CIs but parallels that of PAO1 (Table 3).

In addition, despite its apparent deficiency in QS, CI-5 initiated a strong biofilm (Table 3). The production of both LasB and pyocyanin by P. aeruginosa is stringently controlled by QS (de Kievit & Iglewski, 2000; Rumbaugh et al., 2000). Similarly, QS is required for biofilm formation by P. aeruginosa, including initiation (de Kievit et al., 2001). The mechanism(s) through which CI-5 produces pyocyanin and biofilm initiation (despite the inefficient QS systems) is not known at this time. The key to understanding such a mechanism is to completely characterize CI-5 with respect to the production of other QS-controlled factors including rhamnolipids, alkaline phosphatase and lectins.

**ACKNOWLEDGEMENTS**

This work was supported by the Department of Surgery, TTUHSC. This research was supported in part by a Howard Hughes Medical Institute grant through the Undergraduate Biological Sciences Education Program to Texas Tech University. The authors thank B. Iglewski for the P. aeruginosa strains PDO100 and PAO-1P2. We also thank R. Kolter for P. aeruginosa strain PA14ΔfgK and C. Fuqua for the A. tumefaciens strain NTL4. The authors would also like to thank J. Colmer-Hamood for critical review of the manuscript.

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