Virulence determinants from a cystic fibrosis isolate of *Pseudomonas aeruginosa* include isocitrate lyase

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Chronic lung infections caused by *Pseudomonas aeruginosa* are the leading cause of morbidity and mortality for cystic fibrosis (CF) patients. Adaptation of *P. aeruginosa* to the CF lung results in the loss of acute virulence determinants and appears to activate chronic virulence strategies in this pathogen. In order to identify such strategies, a random transposon mutagenesis was performed and 18 genes that were required for optimal infection of alfalfa seedlings by FRD1, a CF isolate of *P. aeruginosa*, were recognized. The largest subset of genes (seven of the 18), were associated with central carbon metabolism, including the gene that encodes isocitrate lyase (ICL), *aceA*. Because FRD1 is avirulent in animal infection models, we constructed an ICL mutant in *P. aeruginosa* strain PAO1 in order to assess the requirement of ICL in mammalian infection. The PAO1 ICL mutant was less virulent in the rat lung infection model, indicating that ICL is required for the pathogenesis of *P. aeruginosa* in mammals. Furthermore, FRD1 showed increased ICL activity and expression of an *aceA*:lacZ fusion compared to PAO1. We suggest that upregulation of ICL occurred during adaptation of FRD1 to the CF lung and that some of the novel virulence mechanisms employed by FRD1 to infect alfalfa seedlings may be the same mechanisms *P. aeruginosa* relies upon to persist within human niches.

**INTRODUCTION**

Cystic fibrosis (CF) patients are highly susceptible to bronchopulmonary infections with various bacteria. They are especially vulnerable to chronic infections caused by the opportunistic pathogen *Pseudomonas aeruginosa*, and these infections are the leading cause of lung dysfunction and mortality of CF patients. The initial infecting *P. aeruginosa* strain, believed to be acquired from the environment, converts within the CF lung to a phenotype that resists clearing by the host immune system and by therapeutic measures. This conversion correlates with overproduction of alginate by *P. aeruginosa* and with establishment of a chronic infection and increased mortality of CF patients (Henry et al., 1992). Isolates that undergo this conversion are typically maintained within the lungs of the patient for the patient’s entire life span (Mahenthiralingam et al., 1996; Struelens et al., 1993).

Typical *P. aeruginosa* isolates from the environment that infect CF patients produce numerous virulence determinants that contribute to establishing infections in animal model systems (Cox, 1982; Meyer et al., 1996; Nicas & Iglewski, 1985; Ostroff et al., 1989b; Wilderman et al., 2001). However, *P. aeruginosa* recovered from the lungs of chronically infected CF patients typically lack some of these virulence determinants, suggesting that these products are dispensable for long-term maintenance of *P. aeruginosa in vivo* (Dacheux et al., 2001; Luzar & Montie, 1985; Smith et al., 2006; Woods et al., 1986). Conversely, *P. aeruginosa* gains an additional virulence determinant by overproducing the exopolysaccharide alginate within the CF lung. Alginate appears to form a protective barrier around the bacterium and limits exposure to oxidative radicals, antibiotics, opsonizing antibodies and phagocytes (Hatch & Schiller, 1998; Oliver & Weir, 1985; Pedersen et al., 1990; Simpson et al., 1989). In addition, *P. aeruginosa* forms microcolonies encapsulated in alginate and mucus within the CF lung (Worlitzsch et al., 2002). This biofilm mode of growth limits the access of various immune molecules and antibiotics to the bacterium. Biofilm growth may also aid colonization by promoting genetic diversity within the bacterial population (Ehrlich et al., 2005) and by allowing a subset of bacteria to exist in a reduced metabolic state and/or exist under anaerobic conditions, both of which can provide protection from elements of the immune system and antibiotics (Fux et al., 2005).

**Abbreviations:** CF, cystic fibrosis; ICL, isocitrate lyase; PQS, *Pseudomonas* quinolone signal.
It is becoming increasingly clear that microbial pathogens use different sets of virulence determinants and strategies for maintaining chronic infections versus establishing acute infections (Costerton et al., 2003; Hong et al., 2000; Young et al., 2002). Both Mycobacterium tuberculosis and P. aeruginosa can establish decade-long infections in the lungs of humans and these pathogens appear to utilize similar strategies. For example, M. tuberculosis resides in granulomas during chronic lung infections, where it appears to exist in a reduced metabolic state under anaerobic conditions (Young et al., 2002), similar to the existence of P. aeruginosa in biofilms in the CF lung. In addition, both P. aeruginosa and M. tuberculosis appear to alter their carbon metabolic activities while in the human lung (Honer zu Bentrup & Russell, 2001; Silo-Suh et al., 2005). Identification of additional virulence determinants that are active in P. aeruginosa within the CF lung and characterization of the role they play in pathogenesis or survival may provide strategies for treating these and other chronic infections.

To identify chronic virulence determinants, several research groups rely upon chronic animal model systems of infection (Fang et al., 2005; McKinney et al., 2000). We suggest that an alternative approach is to study bacterial isolates that have adapted to a chronic infection lifestyle, such as P. aeruginosa isolates adapted to the CF lung. Some of these adaptations manifest in P. aeruginosa under laboratory conditions because of DNA alterations induced by and selected within the CF lung. Likewise, those virulence determinants that no longer provide an advantage, such as some acute virulence determinants, appear to be readily lost as P. aeruginosa adapts to the CF lung. Therefore, the virulence determinants that are maintained, acquired or altered in P. aeruginosa following adaptation to the CF lung are potential chronic virulence determinants.

We previously determined that FRD1, a typical P. aeruginosa CF isolate, uses novel infection mechanisms compared to the wound isolate PAO1 to infect alfalfa seedlings (Silo-Suh et al., 2002). We suggest that some of the novel virulence mechanisms employed by FRD1 to infect alfalfa may be the same mechanisms it relies upon to persist within the CF lung.

**METHODS**

**Bacterial strains, plasmids and media.** Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, bacteria were cultured in L-broth (LB) or on L-agar. No-carbon E minimal medium (NCE) supplemented with 0.1% (w/v) Casamino acids and with succinate (20 mM) or acetate (20 mM) as a carbon source was used for growth on minimal media (Davis et al., 1980). Data for growth curves were obtained with a Synergy HT plate reader (Bio-Tek Instruments). UV-visible absorption spectra were recorded on a Shimadzu UV-1601 spectrophotometer using 1 cm path-length cells. P. aeruginosa was grown in King’s B medium for poyverdine assays and in 1% (w/v) peptone broth supplemented with 1% (w/v) NaCl and 1% (w/v) glycerol for phospholipase C assays and pyocyanin assays as previously described (Essar et al., 1990). A 1:1 mixture of L-agar and Pseudomonas Isolation Agar (PIA) (PIA) was used to select for P. aeruginosa transconjugants and to counterselect for Escherichia coli following triparental mating. Media were solidified with 1.5% (w/v) Bacto Agar (Difco; Becton Dickinson). Antibiotics were purchased from Sigma-Aldrich and used at the following concentrations: 100 µg ampicillin ml⁻¹ for E. coli; 180 µg carbenicillin ml⁻¹ for P. aeruginosa; 20 µg gentamicin ml⁻¹ for E. coli and 200 µg ml⁻¹ for P. aeruginosa.

**Table 1. P. aeruginosa strains and plasmids**

Abbreviations used for genetic markers are described by Holloway et al. (1979). Ap', ampicillin resistance. Alternative strain names shown in parentheses.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype, relevant characteristics</th>
<th>Source</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td>FRD1</td>
<td>CF isolate, mucoid</td>
<td>Ohman &amp; Chakrabarty (1981)</td>
</tr>
<tr>
<td>PAO1</td>
<td>Wound isolate, nonmucoid</td>
<td>Holloway et al. (1979)</td>
</tr>
<tr>
<td>FRD1 ΔaceA (LS1539)</td>
<td>FRD1 aceA101: :aacCl</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 ΔaceA (LS1545)</td>
<td>PAO1 aceA101: :aacCl</td>
<td>This study</td>
</tr>
<tr>
<td>FRD1 ΔphiS (LS1557)</td>
<td>FRD1 phiS101: :aacCl</td>
<td>This study</td>
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<tr>
<td>FRD1 ΔaceA+ (JH168)</td>
<td>FRD1 aceA complemented for aceA</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 ΔaceA+ (JH166)</td>
<td>PAO1 aceA complemented for aceA</td>
<td>This study</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pAG408</td>
<td>Mini Tn5 containing plasmid</td>
<td>Suarez et al. (1997)</td>
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<tr>
<td>pLS1536</td>
<td>aceA101 in pBluescript K (+)</td>
<td>This study</td>
</tr>
<tr>
<td>pLS1552</td>
<td>phiS101 in pBluescript K (+)</td>
<td>This study</td>
</tr>
<tr>
<td>pJH132</td>
<td>aceA-lacZ transcriptional fusion in pSS223</td>
<td>This study</td>
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<tr>
<td>pJH122</td>
<td>aceA complementing plasmid</td>
<td>This study</td>
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containing transposon insertions. Colonies that grew on gentamicin were replicated onto L-agar supplemented with carbenicillin to identify clones with pAG408 insertions. The clones resistant to both gentamicin and carbenicillin were discarded while the gentamicin-resistant carbenicillin-sensitive clones were characterized further.

**Alfalfa seedling infection assay.** Approximately 1700 independent FRD1 transposition insertion mutants were screened for virulence in the alfalfa seedling infection assay. Seeds of alfalfa variety 57Q77, a wild-type strain not bred for pest resistance, were provided by Pioneer Hi-Bred International. The alfalfa assay was conducted as previously described (Silo-Suh et al., 2002) with the following modifications: FRD1 and derivatives were inoculated onto wounded alfalfa seedlings using $10^8$ c.f.u. per seedling while PAO1 and derivatives were inoculated using $10^4$ c.f.u per seedling. Water agar plates containing inoculated seedlings were sealed with Parafilm and placed in a 30 °C incubator without light. Disease symptoms were scored 6–7 days following inoculation by visual inspection. Seedlings with maceration symptoms were scored positive for infection. Each transposon insertion mutant was initially screened with 10 alfalfa seedlings. Putative mutants that were reduced for virulence on alfalfa by twofold or more compared to the parental strain were rescreened in subsequent rounds of 20 and 40 seedlings. PAO1 and PAO1aceA were inoculated on 60 seedlings for each experiment. Data were expressed as the mean ± standard deviation and analysed for significance using an ANOVA (InStat; Graph Pad Software). A value of $P<0.05$ was considered significant.

**DNA manipulations, transformations and conjugations.** *E. coli* strain DH10B was routinely used as a host strain for cloning. DNA was introduced into *E. coli* by electroporation and into *P. aeruginosa* by conjugation as previously described (Suh et al., 1999). Plasmids were purified with QIAprep Spin Miniprep columns (Qiagen). DNA fragments were excised from agarose gels and purified using the Qiaex II DNA gel extraction kit (Qiagen) according to the manufacturer’s instructions. Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs. Either Pfu from Stratagene or Taq from New England Biolabs was used for PCR amplification of DNA. Oligonucleotides were purchased from Integrated DNA Technologies.

**Southern blot analysis.** Genomic DNA was extracted from FRD1 and derivatives using the Wizard Genomic Extraction kit (Promega), digested with the appropriate restriction enzymes and electrophoretically separated on a 1 % agarose gel. The DNA was transferred to Hybond-N+ membrane (Amersham Pharmacia Biotech) via capillary blotting and fixed by baking for 2 h at 80 °C. The blot was probed with a 800 bp DNA fragment corresponding to the gentamicin resistance gene, which was biotinylated using the Psvoralen-biotin kit (Ambion). Detection of the probe was performed using the BrightStar BioDetect kit (Ambion).

**Identification of transposon insertion sites.** Genomic DNA was prepared from each of the transposon insertion mutants using Wizard Genomic (Promega), digested with the appropriate enzymes, cloned into pBluescript K+ and electroporated into *E. coli* DH10B. The gentamicin-resistant colonies recovered from the transformation were verified for the presence of transposon sequences by PCR and then sequenced using primer LSP368 (cgagggcgtcaattcgaggg). The plasmids containing cloned transposon and flanking *P. aeruginosa* sequences were sequenced by the Auburn University Research and Instrumentation Facility.

**Growth competition.** FRD1 and FRD1 ppsE were inoculated into 12 ml fresh L-broth containing 20 μl of an overnight culture. The mixed culture was sampled for c.f.u on L-agar and L-agar plates containing gentamicin periodically over 24 h.

**Construction of *P. aeruginosa* aceA and phzS mutants.** To generate aceA mutants of *P. aeruginosa*, the suicide plasmid pLS1536 was constructed: a DNA sequence containing ~400 bp upstream and 430 bp downstream of the aceA coding sequence was PCR amplified from FRD1 cells with *Pfu* and cloned into the Smal site of pBluescript K+ (*). The resulting plasmid was digested with *SphI* and the internal 1.3 kb fragment of the aceA coding sequence was removed and replaced with the *aacC1* gene encoding gentamicin resistance as a Smal fragment (Schweizer, 1993). This was followed by introduction of an origin of transfer (oriT) of RP4 on a ~230 bp HindIII fragment (Suh et al., 2004). pLS1536 was introduced into *P. aeruginosa* strains FRD1 and PAO1 by triparental mating, and potential aceA mutants were isolated as gentamicin-resistant carbenicillin-sensitive clones, indicating a double crossover event. Replacement of the wild-type aceA gene with the aceA101::aacC1 allele was verified by PCR analysis.

To construct *P. aeruginosa* phzS mutants, the phzS coding sequence along with ~345 bp upstream and 150 bp downstream sequences was PCR amplified from FRD1 cells with *Pfu* and cloned into the Smal site of pBluescript K+ (*). An aceA1 gene carried on a Smal fragment was cloned into the unique *ScaI* site internal to the phzS coding sequence and then oriT was added as an ~230 bp HindIII fragment. The resulting plasmid, pLS1552, was conjugated into strain FRD1 as described above and potential mutant alleles were isolated as gentamicin-resistant carbenicillin-sensitive clones. Replacement of the wild-type phzS gene with the phzS101::aacC1 allele was verified by PCR.

**Construction of aceA transcriptional fusion and complemented strains.** To construct the aceA::lacZ transcriptional fusion, the PCR fragment containing the aceA gene from FRD1 described above was digested with EcoRI and cloned into the Smal site of pSS223 (Suh et al., 2004). The construct containing the aceA promoter and the 5′ coding sequence in the proper orientation was verified by PCR and restriction digest before it was conjugated into *P. aeruginosa*.

To complement the aceA mutation in cis, aceA was PCR amplified from FRD1 with *Pfu* and cloned into pBluescript K+ (*). An aceA fragment was cloned into the unique *SacI* site of the phzS coding sequence and then oriT was cloned into the *HindIII* site of the phzS coding sequence and then oriT was cloned into the unique *SacI* site of the phzS coding sequence and then oriT was cloned into the unique *SacI* site of the phzS coding sequence. The resulting plasmid, pJH109, was converted to a mobilizable plasmid, pJH122, by the addition of oriT to the plasmid, pJH122 was introduced into *P. aeruginosa* via triparental mating. The complemented FRD1aceA and PAO1aceA mutants were designated FRD1aceA+ (JH168) and PAO1aceA+ (JH166) respectively.

**Biochemical assays.** Alginate was isolated from *P. aeruginosa* culture supernatants that were dialysed against distilled water as previously described (Suh et al., 1999), and the alginate level (i.e. uronic acid) was quantified by the carbazole method (Knutson & Jeanes, 1968) using *Macrocystis pyrifera* alginate (Sigma-Aldrich) as a standard. Pyocyanin was purified and measured from 20 h cultures as described by Essar et al. (1990). Degradation of *p*-nitrophenolphosphorylcholine was used to measure phospholipase C activity as previously described, using 1 mg protein from filtrated supernatant (Suh et al., 1999). Phospholipase C activity was measured as the increase in A405 min$^{-1}$ (mg protein)$^{-1}$. Elastase and protease were measured as previously described (Suh et al., 1999). β-Galactosidase assays were performed as described by Miller (1972). Isochorotic lyase (ICL) was measured according to the Sigma protocol EC 4.1.3.1 with minor modifications. *P. aeruginosa* cells were harvested from stationary cultures, resuspended in TE pH 6.8 and sonicated. Following centrifugation, the quantity of total proteins in the cellfree extracts was determined by the Bradford method (Bio-Rad). Each assay was conducted with 50 μg protein in a final volume of 1 ml.

**Biofilm growth.** Measurement of static biofilm activity was performed as described by Head & Yu (2004). Briefly, *P. aeruginosa* was grown overnight in L-broth, diluted and adjusted to OD$_{600}$=0.5, from which 5 μl was inoculated into 125 μl fresh L-broth in a 96-well

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microtitre plate. The plate was incubated overnight at 37 °C for 15 h prior to staining with crystal violet and optical density readings.

**Rat chronic lung infections.** *P. aeruginosa* strains were tested for their ability to cause respiratory infections in the agar bead model in rats as described by Cash et al. (1979). For each *P. aeruginosa* strain tested, eight male Sprague–Dawley rats weighing 200–220 g (Charles River Breeding Laboratories) were tracheostomized under anaesthesia and inoculated with ~10^6 c.f.u. bacteria embedded in agar beads. On day 14 post-infection, the lungs were aseptically removed. For each set of strains tested, lungs from four animals were homogenized in PBS (Polytron homogenizer, Brinkmann Instruments) and serial dilutions were plated onto trypticase soy agar to determine bacterial counts. The remaining homogenate served as a source of tissue samples for histopathological changes as previously described (Bernier et al., 1987).

The lungs were fixed in 10 % formalin and examined for quantitative histopathological changes as previously described (Bernier et al., 2003). The lung sections were scanned using an Epson 1650 scanner. Areas of inflammation, characterized by cellular infiltration, were identified and digitized with Scion Image software and reported as the percentage of the total area of the lung section that was covered by inflammatory exudates.

**RESULTS**

**Transposon mutagenesis to identify FRD1 mutants defective for virulence on alfalfa seedlings**

To identify virulence strategies of *P. aeruginosa*, a transposon insertion library was generated in strain FRD1 and evaluated. Approximately 99 % of the transposon insertions were carbenicillin sensitive, indicating a low frequency of plasmid insertion. Southern analysis confirmed that a single transposon insertion was present in each of the 21 mutants (data not shown). Loss of the mucoid phenotype and appearance of auxotrophy both occurred at ~1 %, indicating random insertion of the mini-Tn5 in FRD1. The non-mucoid and auxotrophic mutants were discarded. Non-mucoid mutants were likely to contain transposon insertions in the alginate biosynthetic operon or in *algT*, which encodes the alternative sigma factor, sigma-22. Both classes of non-mucoid mutants have previously been tested for virulence in the alfalfa assay (Silo-Suh et al., 2002).

Of ~1700 FRD1 transposon insertion mutants screened in the alfalfa assay, 53 exhibited reduced virulence on alfalfa seedlings. The 21 mutants most severely affected for virulence on alfalfa seedlings were characterized for this investigation (Table 2). With the exception of the FRD1 *pqsB* mutant, all mutants showed a more than twofold reduction in virulence on alfalfa compared to the parental strain. In addition, none of the mutants showed a growth defect in minimal medium with succinate as the carbon source compared to FRD1 (data not shown).

**Identification of transposon insertion sites**

The transposon insertion sites were mapped for all 21 mutants with 18 different genes affected (Table 2). A total of ~1700 FRD1 transposon insertion mutants screened in the alfalfa assay, 53 exhibited reduced virulence on alfalfa seedlings. The 21 mutants most severely affected for virulence on alfalfa seedlings were characterized for this investigation (Table 2). With the exception of the FRD1 *pqsB* mutant, all mutants showed a more than twofold reduction in virulence on alfalfa compared to the parental strain. In addition, none of the mutants showed a growth defect in minimal medium with succinate as the carbon source compared to FRD1 (data not shown).

**Table 2.** FRD1 transposon insertion mutants reduced for virulence on alfalfa

Values represent the percentage of alfalfa seedlings with maceration symptoms following inoculation with bacterial cells of the FRD1 transposon insertion mutant tested. Each value is the mean ± SD of three experiments and is normalized to the parental strain (100 % of alfalfa seedlings with maceration symptoms). All of the mutants are significantly different from the parental strain FRD1 (*P*<0.001).

<table>
<thead>
<tr>
<th>Group by function</th>
<th>Tn insertion site</th>
<th>Infection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PQS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA0997, <em>pqsB</em> β-ketoacyl carrier protein synthase</td>
<td>52 ± 11.10</td>
<td></td>
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<tr>
<td>PA1000, <em>pqsE</em> quinolone signal response protein</td>
<td>23 ± 11.6</td>
<td></td>
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<tr>
<td>PA1000, <em>pqsE</em> quinolone signal response protein</td>
<td>23 ± 10.3</td>
<td></td>
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<tr>
<td>PA1000, <em>pqsE</em> quinolone signal response protein</td>
<td>25 ± 16.4</td>
<td></td>
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<tr>
<td>PA0998, <em>pqsC</em> β-ketoacyl carrier protein synthase</td>
<td>10 ± 8.1</td>
<td></td>
</tr>
<tr>
<td><strong>Carbon metabolism</strong></td>
<td></td>
<td></td>
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<tr>
<td>PA3588, probable porin, <em>opdB</em></td>
<td>27 ± 13.8</td>
<td></td>
</tr>
<tr>
<td>PA3588, probable porin, <em>opdB</em></td>
<td>28 ± 0.8</td>
<td></td>
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<tr>
<td>PA2634, probable isocitrate lyase</td>
<td>10 ± 6.3</td>
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<tr>
<td>PA0794, probable aconitate hydratase</td>
<td>22 ± 14.7</td>
<td></td>
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<tr>
<td>PA2253, <em>ansA</em> L-asparaginase I</td>
<td>30 ± 15.0</td>
<td></td>
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<tr>
<td>PA2637, <em>nuoA</em> NADH dehydrogenase I chain A</td>
<td>3 ± 5.0</td>
<td></td>
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<tr>
<td>PA1066, probable short-chain dehydrogenase</td>
<td>15 ± 10.7</td>
<td></td>
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<tr>
<td>PA4466, probable phosphoryl carrier protein, <em>npr</em></td>
<td>28 ± 15.0</td>
<td></td>
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<tr>
<td><strong>Other</strong></td>
<td></td>
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<tr>
<td>PA5077, <em>mdoH</em> periplasmic glucan biosynthesis</td>
<td>8 ± 7.5</td>
<td></td>
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<tr>
<td>PA5078, <em>mdoG</em> conserved hypothetical protein</td>
<td>10 ± 0.0</td>
<td></td>
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<tr>
<td>PA0011, probable <em>ltrR</em>, lauroyl acyltransferase</td>
<td>28 ± 19.4</td>
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<tr>
<td>PA2503, hypothetical protein</td>
<td>21 ± 14.6</td>
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<tr>
<td>PA4028, probable ATP-dependent RNA helicase</td>
<td>22 ± 9.8</td>
<td></td>
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<tr>
<td>PA4374, probable RND efflux membrane protein</td>
<td>20 ± 14.1</td>
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<td>PA3521, probable <em>tolC</em>, protein secretion</td>
<td>32 ± 8.3</td>
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<tr>
<td>PA4316, <em>sbcB</em> exoDNase I</td>
<td>8 ± 9.6</td>
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**Virulence determinants of CF-associated *P. aeruginosa***

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comparison of the DNA sequences indicates that two groupings of mutants can be distinguished based on functionality. The first group contains transposon insertions in pqs genes, which encode one of three quorum-sensing systems in P. aeruginosa. Three of the mutants contained transposon insertions in pqsE, while one mutant each contained insertions in pqsB and pqsC. All the pqsE insertion mutations mapped to an identical location, suggesting that there is an insertion hot-spot for this gene or that the mutants are genetic siblings. Alternatively, the pqsE mutant might have a growth advantage over the parental strain. However, a competitive growth assay did not support this possibility (data not shown). All three FRD1 pqsE mutants were similarly reduced for virulence on alfalfa (Table 2), indicating that the alfalfa seedling assay is highly reproducible.

The second group of mutants is represented by insertions in seven different genes, all of which potentially play a role in carbon metabolism (Fig. 1). ICL (PA2634), encoded by aceA, is specific to the glyoxylate shunt pathway and allows a variety of organisms to grow on fatty acids or acetate as a sole carbon source. PA0794 encodes an uncharacterized aconitate hydratase that may substitute for AcnA or AcnB in the tricarboxylic acid (TCA) pathway or may function in propionate metabolism as suggested by Grimek & Escalante-Semerena (2004). L-Asparaginase I (PA2253) converts asparagine into aspartate, which is one step away from conversion to oxaloacetate. PA3588 encodes OpdR, a porin of the OpdK subfamily, and is proposed to be involved in transport of phenylacetate, a short-chain fatty acid (Tamber et al., 2006). PA1066 is predicted to encode a short-chain alcohol dehydrogenase involved in fatty acid β-oxidation. The gene nuoA (PA2637) encodes chain A of the proton-pumping NADH dehydrogenase I. Mutations in the nuo operon in E. coli inhibit citrate synthase and malate dehydrogenase of the TCA cycle (Pruss et al., 1994). PA4466 encodes a gene with homology to npr, postulated to link carbon and nitrogen assimilation in E. coli (Powell et al., 1995). Most of the genes in this group affect other cellular functions, including roles in nitrogen metabolism (ansA and npr) and iron homeostasis (npr and aconitase). Further testing will distinguish which of these functions are required for virulence of P. aeruginosa on alfalfa seedlings.

Characterization of the transposon insertion mutants

We characterized the mutants using several biochemical assays to potentially identify additional groupings of mutants and determine whether some acute virulence determinants were active in FRD1 and its derivatives. Although all 18 target mutants were tested in each of the biochemical assays, only the data for the mutants that display a phenotype different from FRD1 are shown.

We first tested for elastase and pyocyanin production because these virulence determinants play a role in animal lung infection models (Blackwood et al., 1983; Elsheikh et al., 1987). Three of the 18 mutations that affected virulence of FRD1 on alfalfa also produced less elastase than FRD1 (Fig. 2a), including the pqsE mutant as expected (P<0.001) (Coin et al., 1997; Diggie et al., 2003). Unexpectedly, neither the FRD1 pqsB nor pqsC mutants showed reduced elastase production, suggesting that the transposon insertions in these genes are not polar on the downstream pqsE gene. In addition, there appears to be a differential requirement for Pseudomonas quinolone signal (PQS) biosynthesis (pqsB and pqsC) versus response to PQS (pqsE) for elastase production.

In particular, pyocyanin is crucial for P. aeruginosa to establish lung infections in mice (Lau et al., 2004). Pyocyanin production was variable in many of the FRD1 mutants reduced for virulence on alfalfa (Fig. 2b). Disruption of the pqs operon, nuoA, PA0428 (putative RNA helicase) and PA0794 (putative aconitate) led to reduced pyocyanin production by FRD1, while disruptions in opdR, PA4466 and sbcB resulted in increased pyocyanin production. These ambiguous results led us to test whether pyocyanin is essential for FRD1 virulence on alfalfa. Disruption of phzS, a gene required for pyocyanin biosynthesis, did not affect the ability of FRD1 to infect alfalfa (data not shown), indicating that lack of pyocyanin production by several of the target mutants was not the cause for loss of virulence.

Phospholipase C cleaves membrane phospholipids and is essential for P. aeruginosa virulence on a variety of host organisms (Berk et al., 1987; Ostroff et al., 1989a; Rahme et al., 1995; Wiener-Kronish et al., 1993). Given that several
of the target mutants are possibly defective in fatty acid catabolism, we predicted a role for phospholipase C in liberating fatty acids for *P. aeruginosa* catabolism during infection. However, only one of the FRD1 targets, PA0428 (RNA helicase), in this study was defective for phospholipase C activity compared to the parental strain (Fig. 3a). Verification of a role for phospholipases in the alfalfa assay will require the generation and testing of mutants that are specifically affected in phospholipase production.

Several of the FRD1 mutants reduced for virulence on alfalfa produced yellow colonies on minimal medium agar, suggesting overproduction of pyoverdine. We confirmed that three of the 18 mutants produced significantly more pyoverdine than FRD1 but less than PAO1 (Fig. 3b). The mechanisms underlying the loss of pyoverdine production by CF isolates of *P. aeruginosa* are unknown. The data presented here suggest that FRD1 is impaired in regulation of pyoverdine production and not biosynthesis. In addition, the uncharacterized aconitase encoded by PA0794 appears to play a role in iron metabolism because disruption of this gene resulted in increased pyoverdine production.

Although all the targets selected for this study were visibly mucoid on solid media, we observed substantial differences in the viscosity of liquid-grown cultures, suggesting alterations in alginate production. As shown in Fig. 3(c), disruption of the PQS biosynthesis genes *pqsB* and *pqsC* reduced alginate production by FRD1, suggesting a relationship between quinolone signalling and alginate biosynthesis. Several genes not previously linked with alginate production were also identified here and include *opdR*, *nuoA* and *aceA*, all three of which are associated with carbon metabolism as indicated above.

**FRD1 virulence mutants are defective for biofilm formation**

*P. aeruginosa* is reported to form biofilms within the lungs of CF patients and this mode of growth likely facilitates persistence of the bacterium within this niche (Werner *et al.*, 2004; Worlitzsch *et al.*, 2002). Although CF isolates can be highly variable with respect to biofilm formation, strain FRD1 can form more robust biofilms than PAO1 under certain conditions (Lee *et al.*, 2005; O’Toole & Kolter, 1998; Pham *et al.*, 2004). We observed increased biofilm formation by FRD1 compared to PAO1 using the biostatic biofilm assay developed by O’Toole & Kolter (1998). Disruptions in *pqsE*, PA0428 (RNA helicase), *opdR*, *nuoA* and *sbcB* resulted in poor biofilm formation by FRD1, while a disruption of *pqsC* enhanced biofilm formation (Fig. 4). Several of the targets described here have not previously been associated with biofilm formation in *P. aeruginosa* and therefore may be specific for the ability of *P. aeruginosa* CF isolates to form biofilms.

**ICL is required for lung infection in rats**

Of all the target genes identified in this report, only *aceA* has been demonstrated to be required for bacterial persistence in animal model systems of infection (Fang *et al.*, 2005; McKinney *et al.*, 2000). To determine whether *aceA* is required for virulence and persistence of *P. aeruginosa*, we generated a mutation in this gene in PAO1, an isolate known to persist in the rat chronic lung infection model. We previously determined that FRD1 is unable to establish an infection in this model system (Silo-Suh *et al.*, 2002). We verified that the disruption of *aceA* rendered PAO1 unable to grow on acetate as a sole carbon source as previously described (data not shown and Diaz-Perez *et al.*, 2007). As shown in Table 3, the PAO1 *aceA* mutant was significantly reduced for virulence on alfalfa seedlings. More importantly, disruption of *aceA* in PAO1
led to a fourfold reduction in histopathology in rat lungs, indicating that ICL is required for optimal virulence of P. aeruginosa in mammals (Table 3, Fig. 5).

ICL activity is upregulated in FRD1

In order to address whether aceA functions similarly in FRD1 and PAO1, we tested cell-free extracts derived from these isolates for ICL activity. Unexpectedly, FRD1 cell-free extracts showed increased ICL activity (approx. 12-fold) compared to PAO1 extracts (Fig. 6a). The low ICL activity observed for PAO1 growing in L-broth is consistent with published results that aceA is specifically induced by carbon sources that utilize the glyoxylate pathway, such as fatty acids and acetate (Diaz-Perez et al., 2007; Honer Zu Bentrup et al., 1999). As expected, the aceA mutants of each parental strain were deficient for ICL and this phenotype was complemented in cis by the introduction of plasmid pJH122 containing a wild-type copy of aceA derived from FRD1 (Fig. 6a). The FRD1 and PAO1 aceA mutants were restored to approximately wild-type activity, indicating that the FRD1 aceA gene functioned comparably in each background.

To address whether the altered ICL activity in FRD1 was caused by increased transcriptional activation, an aceA::lacZ fusion was constructed and introduced into both wild-type P. aeruginosa isolates. The relative expression of β-galactosidase from the aceA::lacZ fusion paralleled the activity of ICL under the same growth conditions in both FRD1 and PAO1, indicating that the increased ICL activity in FRD1 was related to increased transcriptional activity of aceA (Fig. 6b).

Fig. 3. FRD1 target mutants altered in phospholipase C, pyoverdine and alginate. The FRD1 target mutants are designated by their transposon insertion site. Values represent the mean ± SD of three experiments. (a) Phospholipase C. (b) Pyoverdine. (c) Alginate.

Fig. 4. FRD1 target mutants altered in biofilm formation, measured as A570. The FRD1 target mutants are designated by their transposon insertion site. Values are representative of three experiments and are the mean ± SD of six wells per experiment.

Fig. 5. Scans of representative lung sections from animals infected with (a) PAO1 and (b) PAO1aceA. Regions with darker staining are indicative of areas of greater inflammation. The percentages of lung section area with inflammatory exudates are 44.3 % and 40.1 % for the sections shown in (a), and 12.4 % and 9.2 % for the sections shown in (b).
DISCUSSION

Available data suggest that chronic colonizers of the human body invoke novel strategies for avoiding elimination by the host immune system and human intervention. Thus, a better understanding of these strategies is a prerequisite for developing effective methods for treating these diseases. We expect that characterizing the strategies utilized by \textit{P. aeruginosa} to cause chronic respiratory infections in CF patients will provide vital clues to treating these infections and possibly other chronic lung diseases that are affected by microbial infections.

In this study we identified genes required for FRD1, a CF isolate of \textit{P. aeruginosa}, to infect alfalfa seedlings. On the basis of our earlier observations that FRD1 invokes novel strategies to infect alfalfa seedlings compared to the non-CF isolate PAO1, we believe some of these strategies to be important for the persistence of \textit{P. aeruginosa} within the CF lung. Although our mutagenesis and screening strategy did not reach saturation of the \textit{P. aeruginosa} genome, it does provide a glimpse of some of the virulence mechanisms functioning in FRD1. These include the glyoxylate pathway, biofilm activity and quorum sensing mediated by PQS.

We are especially intrigued by the large number of mutations affecting carbon metabolism because of the implications regarding \textit{P. aeruginosa}'s metabolism in the host and the potential avenues for therapeutic intervention. The requirement for ICL by FRD1 and PAO1 to infect alfalfa seedlings and by PAO1 to infect rat lungs indicates that the glyoxylate pathway plays an important role during \textit{P. aeruginosa} pathogenesis. In a recent publication, lung surfactant lipids and amino acids were identified as carbon sources utilized by \textit{P. aeruginosa} within the CF lung as indicated by high \textit{in vivo} expression of the genes encoding catabolism or uptake of these compounds (Son \textit{et al.}, 2007). While considerable data previously supported that peptides and amino acids fuelled the growth of \textit{P. aeruginosa} within the CF lung (Barth & Pitt, 1996; Palmer \textit{et al.}, 2005), our study supports Son \textit{et al.} (2007) in that fatty acids are also important carbon sources for \textit{P. aeruginosa} during chronic infection. The CF lung is rich in various fatty acids, some of which play important roles in lung function, including prostaglandins and phosphatidyl-

Table 3. Isocitrate lyase is required for \textit{P. aeruginosa} virulence on alfalfa and in the rat lung model

<table>
<thead>
<tr>
<th>Strain</th>
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<th>Rat lung infection</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mean bacteria (c.f.u. ml$^{-1}$ per lung)$^\dagger$</td>
<td>Mean % inflammation$^\ddagger$</td>
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</tr>
<tr>
<td>PAO1</td>
<td>77 ± 17.5</td>
<td>4.54 × 10$^5$ ± 5.96 × 10$^5$</td>
<td>44.6 ± 7.84 (38.2–55.7)</td>
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<td>PAO1 aceA</td>
<td>26 ± 12.1</td>
<td>5.12 × 10$^4$ ± 3.34 × 10$^4$</td>
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$^\dagger$Percentage of alfalfa seedlings with maceration symptoms (mean ± SD). The data are representative of two experiments. Significantly different from PAO1 ($P<0.001$).

$^\ddagger$Number of bacteria recovered from rats’ lungs. Values are mean ± SD for four animals at 14 days post-infection. Numbers in parentheses represent the range.

$^\ddagger$Significantly different from PAO1 ($P<0.001$, t-test). Values are mean ± SD for four animals (range in parentheses) at 14 days post-infection.

### Table 3

Isocitrate lyase is required for \textit{P. aeruginosa} virulence on alfalfa and in the rat lung model

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**Fig. 6.** Activity and expression of ICL is altered in FRD1. (a) ICL activity; one unit catalyses the formation of 1.0 µmol glyoxylate min$^{-1}$. The data represent the mean ± SD of four experiments. (b) β-Galactosidase activity. The data represent the mean ± SD of two experiments conducted in duplicate. ○, FRD1; ●, PAO1.
choline. Catabolism of such compounds might facilitate colonization of the CF lung by *P. aeruginosa* by impairing the functions of these compounds.

Also in agreement with Son et al. (2007), we report that aceA is more highly expressed in CF *P. aeruginosa* compared to non-CF isolates. However, our current data suggest that overexpression of aceA in FRD1 is due to aberrant regulation, most likely due to loss of a negative regulator. Consistent with this hypothesis is the observation that the FRD1 aceA gene complemented PAO1aceA to wild-type levels of ICL, suggesting that the FRD1 aceA gene is unaltered. Increased expression of aceA in FRD1 is a likely consequence of adaptation to the CF lung and, as such, expected to facilitate *P. aeruginosa*’s ability to maintain infection within the CF lung. Our current studies raise the possibility of controlling *P. aeruginosa* infections within the CF lung with the use of drugs that inhibit ICL. The apparent absence of this enzyme in humans makes it an attractive therapeutic target.

Interestingly, several other genes we report here to be required for FRD1 virulence on alfalfa were shown to be either upregulated in *P. aeruginosa* within the CF lung, or constitutively expressed in *P. aeruginosa* CF isolates by Son et al. (2007). These genes include nuoA and PA4466 (npr). Therefore, the results of the alfalfa assay agrees with the conclusion of Son et al. (2007) that some highly expressed genes within *P. aeruginosa* CF isolates play important roles during infection.

The second recognized grouping of mutants reduced for virulence on alfalfa were shown to be either upregulated in *P. aeruginosa* within the CF lung, or constitutively expressed in *P. aeruginosa* CF isolates by Son et al. (2007). These genes include *nuoA* and PA4466 (*npr*). Therefore, the results of the alfalfa assay agrees with the conclusion of Son et al. (2007) that some highly expressed genes within *P. aeruginosa* CF isolates play important roles during infection.

In addition to the two functional groupings of mutants recognized here, several of the mutants reduced for virulence on alfalfa have transposon insertions in genes that affect the envelope and outer-membrane properties of *P. aeruginosa*. These targets include two genes predicted to encode multidrug efflux proteins (PA3521 and PA4374) and PA0011, which is predicted to encode a lauroyl transferase with homology to HtrB. This protein functions in lipid A biosynthesis and is required for virulence by several human pathogens, including *Haemophilus influenzae* and *Salmonella enterica* serovar Typhimurium (DeMaria et al., 1997; Jones et al., 1997). Included in this group are the two mutants with transposon insertions in *mdoH* and *mdoG*, which encode membrane-derived oligosaccharides (MDOs). MDOs are branched substituted β-glucan chains present in the periplasm of various Gram-negative bacteria and their expression increases in low-osmolarity medium (Geiger et al., 1992). The MDO mutants tend to suffer pleiotropic effects, most likely due to altered membrane properties, which may account for the requirement for MDOs by a variety of plant and animal pathogens for virulence (Bhagwat et al., 2004; Page et al., 2001). Loss of MDOs can impair growth in hypo-osmotic media, alter chemotaxis and motility, alter porin composition and increase sensitivity to biliary salts (Fiedler & Rötter, 1988).

Six of the FRD1 transposon mutants reduced for virulence on alfalfa were affected in their ability to form static biofilms. Such a large grouping of mutants suggests a continuing role for biofilm formation, or maintenance, in chronic *P. aeruginosa* infections. Continued screening for chronic virulence determinants from CF isolates of *P. aeruginosa* will likely identify additional genes with important roles in persistence of this bacterium in eukaryotic hosts.

**ACKNOWLEDGEMENTS**

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