Influence of RpoN on isocitrate lyase activity in Pseudomonas aeruginosa

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Pseudomonas aeruginosa is the major aetiological agent of chronic pulmonary infections in patients with cystic fibrosis (CF). The metabolic pathways utilized by P. aeruginosa during these infections, which can persist for decades, are poorly understood. Several lines of evidence suggest that the glyoxylate pathway, which utilizes acetate or fatty acids to replenish intermediates of the tricarboxylic acid cycle, is an important metabolic pathway for P. aeruginosa adapted to the CF lung. Isocitrate lyase (ICL) is one of two major enzymes of the glyoxylate pathway. In a previous study, we determined that P. aeruginosa is dependent upon aceA, which encodes ICL, to cause disease on alfalfa seedlings and in rat lungs. Expression of aceA in PAO1, a P. aeruginosa isolate associated with acute infection, is regulated by carbon sources that utilize the glyoxylate pathway. In contrast, expression of aceA in FRD1, a CF isolate, is constitutively upregulated. Moreover, this deregulation of aceA occurs in other P. aeruginosa isolates associated with chronic infection, suggesting that high ICL activity facilitates adaptation of P. aeruginosa to the CF lung. Complementation of FRD1 with a PAO1 clone bank identified that rpoN negatively regulates aceA. However, the deregulation of aceA in FRD1 was not due to a knockout mutation of rpoN. Regulation of the glyoxylate pathway by RpoN is likely to be indirect, and represents a unique regulatory role for this sigma factor in bacterial metabolism.

INTRODUCTION

Bronchopulmonary infections caused by Pseudomonas aeruginosa are the leading cause of mortality for cystic fibrosis (CF) patients. These infections resist eradication by antibiotic therapy and the host immune system, and indicate a need for novel therapeutic strategies. The ability of P. aeruginosa to maintain decade-long infections within the CF lung is attributed in part to virulence mechanisms that evolve as the bacterium adapts to this environment (Lindsey et al., 2008; Nguyen & Singh, 2006). Although P. aeruginosa is nutritionally versatile, within human niches it must adapt to the availability of host-derived nutrients. Within the lungs of CF patients, these nutrients are probably contained in sputum.

The composition of CF sputum is complex. It contains host and bacterial cells, as well as various host- and bacterial-derived compounds (Hoiby, 1998). Transcriptome studies have indicated that PAO1, a wound isolate of P. aeruginosa, primarily uses amino acids as a carbon source when grown in CF sputum (Palmer et al., 2005; Son et al., 2007). In contrast, a CF isolate of P. aeruginosa uses amino acids and lipids (Palmer et al., 2005; Son et al., 2007). The different carbon utilization patterns by these isolates suggest that P. aeruginosa alters its metabolic pathways during chronic infection of the CF lung. This is supported by the observation that regulatory control of several central metabolic enzymes is altered in FRD1, a CF isolate, compared with PAO1 (Lindsey et al., 2008; Silo-Suh et al., 2005).

In a previous study, we exploited the alfalfa seedling model system of infection to identify virulence determinants from P. aeruginosa isolates adapted to the CF lung. Our analysis revealed that the aceA gene, which encodes isocitrate lyase (ICL), is critical for infection of alfalfa seedlings and rat lungs by P. aeruginosa (Lindsey et al., 2008). ICL is one of the two key enzymes of the glyoxylate pathway, which is utilized by some bacteria when growing on fatty acids or acetate as the sole carbon source. Our previous study also demonstrated that aceA expression is upregulated in the CF isolate FRD1, but not in the acute isolate PAO1, when grown in L-broth, a peptide-rich medium. Therefore, aceA gene expression responds to different environmental signals in FRD1 compared with PAO1. Alternatively, aceA may be constitutively expressed in FRD1. In this study, we compared the regulation of aceA expression and ICL activity in PAO1 and FRD1 in response to various carbon sources, in order to better understand the metabolic alterations associated with chronic infections. In addition, we determined that RpoN negatively regulates aceA in P. aeruginosa.

Abbreviations: CF, cystic fibrosis; ICL, isocitrate lyase; LB, L-broth; NCE, no-carbon-E minimal medium.

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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 (both Fischer) at 37 °C. Glutamine (5 mM) was added to cultures to support growth of rpoN mutants, as described by Hendrickson et al. (2001). No-carbon-E minimal medium (NCE) supplemented with 0.1% (w/v) Casamino acids was used to assay for growth on minimal medium (Davis et al., 1980). Additional carbon sources were used at the following concentrations: palmitic acid, 2.5 mM; heptanoic acid, 5 mM; valeric acid, 10 mM. All amino acids were used at 1%, except histidine and tryptophan at 0.5%, and tyrosine at 0.1%. Ethanol was used at 0.5%. The remaining carbon sources were used at 20 mM.

UV-Vis absorption spectra were recorded on a Shimadzu UV-1601 spectrophotometer using cells with 1 cm path length. A 1:1 mixture of L-agar and Pseudomonas isolation agar (Difco) 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). Antibiotics were purchased from Sigma-Aldrich, and used at the following concentrations: 100 μg ampicillin ml⁻¹ for E. coli; 100 μg carbenicillin ml⁻¹ for P. aeruginosa; 20 or 200 μg gentamicin ml⁻¹ for E. coli or P. aeruginosa; 20 or 100 μg tetracycline ml⁻¹ for E. coli or P. aeruginosa.

Table 1. Bacterial strains and plasmids

Abbreviations used for genetic markers are described by Holloway et al. (1979). Alternative strain names are 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>Strain</td>
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<tr>
<td>FRD1</td>
<td>CF isolate, mucoid</td>
<td>Ohman &amp; Chakrabarty (1981)</td>
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<tr>
<td>PAO1</td>
<td>Wound isolate, non-mucoid</td>
<td>Holloway et al. (1979)</td>
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<td>idcR101 in pBluescript K⁺</td>
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<td>pLS574</td>
<td>rpoN101 in pBluescript K⁺</td>
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<td>Plasmid with regulatable promoter</td>
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<td>pUC19 with morT at HindIII</td>
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<td>pJH202</td>
<td>pLAFR1 with PAO1 genomic DNA</td>
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<td>pLS1859</td>
<td>rpoN::lacZ transcriptional fusion in pSS223</td>
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DNA manipulations, transformations and conjugations. E. coli strain DH10B was used routinely as a host strain for cloning. DNA was introduced into E. coli by electroporation, and into P. aeruginosa by conjugation, as described previously (Suh et al., 1999). Plasmids were purified with Qiagen 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.

Construction of P. aeruginosa mutants, and rpoN::lacZ transcriptional fusion. To generate iclR mutants of P. aeruginosa, the suicide plasmid pLS1653 was constructed. Briefly, a DNA fragment containing approximately 300 bp upstream and approximately 250 bp downstream of the iclR-coding sequence (PA0236) was amplified by PCR from FRD1 cells by using Pfu, and cloned into the Smal site of pBluescript K⁺. The resulting plasmid was digested with Smal and MseI, and the internal 160 bp fragment of the iclR-coding sequence was removed, and replaced with the Smal fragment encoding the aacCI gene (Schweizer, 1993). This was followed by introduction of an origin of transfer (oriT) of RP4 on HindIII

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fragment of approximately 230 bp (Suh et al., 2004). pLS1653 was introduced into \textit{P. aeruginosa} strain PAO1 by triparental mating, and potential \textit{idcR} mutants were isolated as gentamicin-resistant carbene-

cillin-sensitive colonies that resulted from a double cross-over event. Replacement of the wild-type \textit{idcR} gene with the \textit{idcR101::aacCI} allele was verified by PCR analysis.

The \textit{P. aeruginosa} \textit{retS} mutant was constructed by PCR amplification (forward primer 5'-tggtgaggttggggtcttc and reverse primer 5'-
tggtgaggttggggtcttc) of an internal 1.1 kb \textit{retS}-coding sequence from FRD1 cells by using \textit{Pfu} polymerase, and it was cloned into the \textit{Smal} site of plBluescript K II suicide plasmid pLS574. The \textit{P. aeruginosa} \textit{rpoN} gene with the coding sequence of approximately 200 bp of the upstream sequence and 1169 bases of \textit{rpoN} was cloned into the EcoRV site internal to the 1.1 kb fragment, and then \textit{oriT} was added as a HindIII fragment of approximately 230 bp. The resulting plasmid, pLS1641, was conjugated into strain PAO1, as described above, and potential mutants were isolated as gentamicin-resistant carbeneillin-sensitive clones. Replacement of the wild-type \textit{retS} gene with the \textit{retS::aacCI} allele was verified by PCR analysis.

\textit{P. aeruginosa} \textit{rpoN} mutants were generated by constructing the suicide plasmid pLS574. The \textit{rpoN} coding sequence was amplified by PCR by using \textit{Pfu} polymerase from PAO1. The PCR fragment included approximately 200 bp of the upstream sequence and 1169 bases of the coding sequence of \textit{rpoN}, and it was cloned into the BamHI–

\textit{EcoRI} site of plBluescript K II. A tetracycline cassette, isolated from pHP450Tc (Fellay et al., 1987), was isolated as a \textit{Smal} fragment, and it was cloned into the internal \textit{SfoI} site of the \textit{rpoN}-coding sequence, and an \textit{oriT} was added as an \textit{EcoRI} fragment. Following conjugation, tetracycline-resistant transconjugants were selected on agar plates containing 5 mM glutamine, and then screened for sensitivity to carbencillin. Replacement of the wild-type \textit{rpoN} gene with the \textit{rpoN::tetTc} allele was verified by PCR analysis. To complement the \textit{rpoN} mutation in \textit{cis}, the \textit{rpoN}-coding sequence was amplified by PCR from both FRD1 and PAO1 by using \textit{Pfu} polymerase, and cloned adjacent to the regulatable promoter \textit{P}_{\text{27}} present on pS5213 (Suh et al., 2004). Constructs were verified by PCR and restriction digests. An \textit{moriT} gene isolated from pLS214 was then cloned as a \textit{HindIII} fragment to generate pLS1785 (containing FRD1 \textit{rpoN}) and pH264 (containing PAO1 \textit{rpoN}). The plasmids were introduced into the FRD1\textit{rpoN} and PAO1\textit{rpoN} mutants via conjugation.

To construct the \textit{rpoN::lacZ} transcriptional fusion, the PCR fragment containing the \textit{rpoN} gene from FRD1, containing 342 bp upstream of the coding sequence and 392 bp of the coding sequence, was cloned into the \textit{Smal} site of pS5223 (Suh et al., 2004). The construct containing the \textit{rpoN} promoter and the \textit{S'} coding sequence in the proper orientation was verified by PCR and restriction digest before it was conjugated into \textit{P. aeruginosa}.

**Identification of \textit{aceA::lacZ} regulator.** To complement FRD1 carrying \textit{aceA::lacZ} (strain \textit{JH132}) for \textit{\beta}-galactosidase activity, a PAO1 library was introduced into \textit{JH139} via triparental mating (Goldberg & Ohman, 1984). From approximately 1000 transconjugants tested for \textit{\beta}-galactosidase activity, three clones partially restored \textit{aceA} regulation in FRD1. Plasmids were isolated from each transconjugant, and restriction digestion indicated that two of the plasmids were identical. Therefore, the two plasmids showing different restriction patterns were mutagenized in \textit{vitro} using EZ-Tn5 (Epigenetec), and transformed into \textit{E. coli}. The pool of mutants was conjugated into FRD1 carrying the \textit{aceA::lacZ} fusion, and transconjugants were screened for \textit{\beta}-galactosidase activity. Plasmids unable to complement FRD1 for \textit{aceA} expression were isolated and characterized to map the transposon insertion sites.

**Biochemical assays.** \textit{\beta}-Galactosidase assays were performed as described by Miller (1972). ICL activity was assayed as described previously (Lindsey et al., 2008), and presented as \(\Delta A_{340} \text{ min}^{-1} \text{ (mg protein)}^{-1}\).

**RESULTS**

**High ICL activity occurs in \textit{P. aeruginosa} CF isolates**

We have demonstrated previously that \textit{aceA} expression and ICL activity are upregulated in the \textit{P. aeruginosa} CF isolate FRD1 compared with the wound isolate PAO1, when grown in LB (Lindsey et al., 2008). The low ICL activity observed for PAO1 under these conditions is consistent with published reports that \textit{aceA} is specifically induced by carbon sources that utilize the glyoxylate pathway (Diaz-Perez et al., 2007; Honer Zu Bentrup et al., 1999). The abnormally high ICL activity observed for FRD1 grown in LB lacking inducible carbon sources may be a consequence of \textit{P. aeruginosa} adaptation to the CF lung. If so, then other CF isolates of \textit{P. aeruginosa} should show a similar phenotype. As shown in Fig. 1, all of the clinical (ten) and environmental (eight) isolates of \textit{P. aeruginosa} tested showed low ICL activity similar to that of PAO1, when grown in LB. In comparison, three of ten CF isolates tested showed high ICL activity that was similar to that of FRD1 under the same growth conditions. While not all of the CF isolates showed deregulated ICL, this phenotype appears to be more common in CF isolates versus \textit{P. aeruginosa} isolates from other sources. Unfortunately, lack of patient history for all of the \textit{P. aeruginosa} CF isolates tested here prevented generation of a correlation between infection duration and emergence of the deregulated ICL phenotype. In addition, there was no correlation between the mucoid phenotype caused by overproduction of alginate and high ICL activity. Out of the 11 CF isolates tested for ICL activity, six were mucoid, and five were non-mucoid. Of the four isolates that showed high ICL activity, two were mucoid (FRD1 and DO5), and two were non-mucoid (DO11 and DO108).

**Effect of carbon sources on \textit{aceA} expression and ICL activity**

In various bacteria, \textit{aceA} expression is regulated by the presence of certain carbon sources, such as acetate and fatty acids, in the growth medium that yield acetyl-CoA during catabolism. We have determined previously that ICL activity and \textit{aceA} expression are upregulated in FRD1 compared with PAO1 when grown in LB (Lindsey et al., 2008). This was an unexpected result because a peptide-rich medium had not previously been shown to induce \textit{aceA} expression in bacteria. To more accurately determine the effect of carbon sources on \textit{aceA} transcription, expression studies have been carried out on FRD1 and PAO1 carrying an \textit{aceA::lacZ} fusion (Lindsey et al., 2008).

We initiated this analysis by testing the effect of several tricarboxylic acid (TCA) cycle intermediates and compounds known to induce \textit{aceA} in \textit{P. aeruginosa} (Diaz-Perez et al., 2007; Kretzschmar et al., 2008). Consistent with published reports, expression of \textit{aceA} in PAO1 was activated only when known inducers, including ethanol,
acetate and various fatty acids, served as the sole carbon source (Fig. 2a). In contrast, expression of aceA was activated in FRD1 in all the carbon sources tested, suggesting that expression of this gene is largely deregulated in FRD1. However, variations in aceA expression levels in response to the different carbon sources suggest that some regulatory controls are active in FRD1.

ICL enzymic activity closely correlated with aceA promoter activity in PAO1 for most of the carbon sources tested, with the notable exceptions of palmitic acid and heptanoic acid (Fig. 2b). For these two fatty acids, the correlation was reversed for unknown reasons. Although ICL activity was consistently high in FRD1 compared with PAO1 in response to the given carbon sources, enzyme activity did not strictly mirror aceA expression in this background. However, ICL activity in FRD1 was highest following growth on carbon sources that strongly induced aceA::lacZ in PAO1. Taken together, the results suggest that ICL activity in both backgrounds is affected by post-transcriptional regulation.

**ICL is induced by ketogenic amino acids in PAO1**

Current reports suggest that *P. aeruginosa* utilizes amino acids and some fatty acids as carbon sources while growing in the CF lung (Palmer *et al.*, 2005; Son *et al.*, 2007). Although the glyoxylate pathway is known to be induced in various bacteria by growth on fatty acids, much less is known about the role of the glyoxylate pathway during catabolism of amino acids. Some ketogenic amino acids yield acetyl-CoA during catabolism, and would require the glyoxylate pathway for catabolism. As expected, several of the ketogenic amino acids, including leucine, isoleucine and tyrosine, induced aceA expression in PAO1 (Fig. 3). For unknown reasons, tyrosine induced aceA more strongly than the other amino acids tested, and expression was highly variable. PAO1 cultures grown on succinate plus tyrosine showed ICL activity similar to that of growth on succinate alone (2500 Miller units). The results confirm reports that ICL expression is subjected to catabolite repression by TCA cycle intermediates (Diaz-Perez *et al.*, 2007).

**ICL regulation**

In an attempt to identify the mechanism of aceA deregulation in FRD1, we measured aceA expression in *P. aeruginosa* derivatives that were disrupted for known regulators of ICL, carbon catabolism or chronic virulence. Because Crc functions to regulate carbon catabolism in *Pseudomonas* spp., we examined the effect that disruption of crc had on aceA expression and ICL activity in PAO1. Crc is involved in repression of multiple carbon catabolic pathways in *P. aeruginosa* when preferred substrates are present (Collier *et al.*, 1996), but it did not appear to control expression of aceA in PAO1 (Fig. 4) or ICL activity (data not shown). Disruption of an ORF (PA0236) predicted to encode IclR, which is a repressor protein for
aceA in various bacteria (Gui et al., 1996), also did not affect aceA expression in PAO1. Finally, we tested the effect of AlgT (AlgU) and RetS, which are two proteins that have been characterized as regulators of chronic virulence in P. aeruginosa, on aceA expression. AlgT, an alternative sigma factor, becomes active in P. aeruginosa during colonization

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**Fig. 2.** Effect of various carbon sources on aceA::lacZ expression and ICL activity. *P. aeruginosa* cultures were grown overnight in NCE supplemented with the given carbon sources. (a) β-Galactosidase activity. (b) ICL activity normalized to protein concentration. Values are the mean (±SD) of two experiments conducted in duplicate. FRD1, open bars; PAO1, hatched bars. CAA, Casamino acids; EtOH, ethanol.

**Fig. 3.** Effect of various amino acids as carbon sources on aceA::lacZ in PAO1. *P. aeruginosa* cultures were grown overnight in NCE supplemented with the given amino acids as carbon sources. Values presented are the mean (±SD) of two experiments conducted in duplicate.

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of the CF lung via acquired mutations in the anti-sigma factor MucA, and is responsible for overproduction of the exopolysaccharide alginate (Xie et al., 1996). In contrast, RetS downregulates genes in PAO1 predicted to be involved in chronic virulence (Goodman et al., 2004). As shown in Fig. 4, AlgT is not responsible for the high aceA activity in the FRD1 background, and RetS appears to positively regulate aceA in PAO1. Because deregulation of aceA::lacZ was not observed in the PAO1 knockout mutants for icdR, crc and retS, it is unlikely that these regulators are the source of aceA deregulation in FRD1. Therefore, we did not test the FRD1 mutant derivatives of the indicated regulators for their effect on aceA::lacZ. In summary, a predictive approach to identify regulators of aceA transcription in P. aeruginosa failed to identify the source of deregulated ICL activity in FRD1.

To identify a putative regulator of aceA expression in FRD1, we complemented FRD1 carrying the aceA::lacZ (strain JH132) for β-galactosidase activity using a PAO1 library, which was introduced into JH139 via triparental mating. Of approximately 1000 transconjugants tested for β-galactosidase activity, we identified three transconjugants with decreased aceA::lacZ expression. Plasmids were recovered from these three transconjugants, and reintroduced into JH139 to verify for genetic complementation of aceA::lacZ expression (Fig. 5a). In order to identify the complementing gene, the plasmids were mutagenized in vitro with EZ-Tn5, and the pool of mutants was transformed into E. coli DH10B. The pool of E. coli transformants was subsequently conjugated into JH139, and then screened for loss of complementation activity that resulted in restoration of high expression of aceA::lacZ. Two plasmid mutants were recovered that had lost complementing activity for the aceA::lacZ fusion (Fig. 5a). Tn5 mapping revealed that, in both plasmids, the transposon had inserted into rpoN. To verify our genetic complementation data that RpoN (sigma-54) regulates aceA in P. aeruginosa, we disrupted rpoN in both PAO1 and FRD1, and then measured ICL activity. The PAO1rpoN mutant showed increased ICL activity compared with PAO1, while FRD1 and the FRD1rpoN mutant showed similar ICL activity (Fig. 5b). In addition, low ICL activity was restored to the PAO1rpoN mutant following complementation with a wild-type copy of rpoN from either FRD1 or PAO1 in cis. Furthermore, compared with PAO1, transcription of the aceA::lacZ fusion in the PAO1rpoN mutant increased steadily over a growth cycle in cultures grown in LB (Fig. 5c). The results are consistent with a negative regulatory effect of sigma-54 on ICL activity by affecting aceA transcription in PAO1. However, expression of aceA::lacZ was much higher in FRD1 compared with the PAO1rpoN mutant (Fig. 5b). This suggests that deregulation of RpoN-mediated repression is not solely responsible for high expression of aceA in FRD1. To confirm that our data were not due to differential expression of rpoN between PAO1 and FRD1, we analysed rpoN::lacZ expression in both strains. As shown in Fig. 5(d), the rpoN::lacZ transcriptional fusion was expressed at a slightly lower level in FRD1 compared with PAO1 throughout a growth cycle, and therefore it is unlikely to account for the ninefold difference in ICL activity between the two isolates. DNA sequencing and complementation of PAO1rpoN mutant with FRD1rpoN demonstrated that rpoN was not altered in FRD1, and that it encoded a functional sigma factor.

**DISCUSSION**

In order to develop effective strategies to disrupt pathogen nutrient acquisition, it is essential to gain a better understanding of these processes. In this study, we characterized regulatory control of the gene encoding ICL in P. aeruginosa. ICL is the first enzyme of the glyoxylate pathway, which allows growth of some bacteria on C₂ compounds, including fatty acids. Such compounds are present in the CF lung along with amino acids, which appear to be major carbon sources for P. aeruginosa in this environment (Barth & Pitt, 1996; Palmer et al., 2005; Son et al., 2007). However, the presence of amino acids in the CF lung is expected to cause catabolite repression of the glyoxylate pathway in P. aeruginosa, according to our findings. In support of this, the glyoxylate pathway is not induced in a non-CF isolate strain, PAO1, growing on CF sputum (Hendrickson et al., 2001). In contrast, the glyoxylate pathway is induced in CF P. aeruginosa growing in CF sputum, suggesting that adaptation to the CF lung environment alters regulation of the glyoxylate pathway for the bacterium (Son et al., 2007). In addition, we showed here

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![Fig. 4. Effect of various mutations on aceA::lacZ expression in P. aeruginosa. β-Galactosidase activity was assayed from overnight cultures of P. aeruginosa grown in LB. Values represent the mean (±sd) of two experiments conducted in duplicate. FRD1 and FRD1algT were grown at 32 °C, while PAO1 and derivatives were assayed using cultures grown at 37 °C.](image-url)
that some *P. aeruginosa* derivatives, including FRD1, adapted to the CF lung constitutively express aceA. Taken together, ICL activity may not be required solely for catabolism of carbon sources in the CF lung, but may benefit the bacterium in other ways. Consistent with this possibility, both alginate and hydrogen cyanide production are reduced in an FRD1 aceA mutant (Hagins et al., 2009; Lindsey et al., 2008). Alginate protects *P. aeruginosa* during chronic infections by limiting the diffusion of oxidative radicals, antibiotics, opsonizing antibodies and phagocytes (Hatch & Schiller, 1998; Oliver & Weir, 1985; Pedersen et al., 1990; Simpson et al., 1989). The role of hydrogen cyanide in *P. aeruginosa* pathogenesis is less clear because a requirement for this product in a mammalian model of infection has yet to be shown. Nevertheless, cyanide has been detected in the sputum of CF patients harbouring *P. aeruginosa*, and cyanide levels are associated with impaired lung function (Ryall et al., 2008; Sanderson et al., 2008). Elevated production of alginate and hydrogen cyanide are associated with *P. aeruginosa* isolated from chronically infected CF lungs (Carterson et al., 2004; Wozniak & Ohman, 1994). Decreased production of these virulence determinants in an FRD1 aceA mutant suggests a correlation between the glyoxylate pathway and persistence of the bacterium in CF lungs. One of the well-defined characteristics of CF isolates of *P. aeruginosa* is the activation of the alternative sigma

![Fig. 5. Effect of rpoN mutation on ICL activity and aceA expression. Values are the mean (± SD) of two experiments conducted in duplicate. (a) *P. aeruginosa* isolates carrying the aceA::lacZ fusion were grown overnight in LB. FRD1C, FRD1 complemented for regulation of aceA with pLAFR1 containing PAO1 genomic DNA (designated pJH202); FRD1C−, FRD1 containing a pJH202 with a transposon insertion in rpoN. (b) ICL activity assayed from overnight cultures of *P. aeruginosa* grown in LB supplemented with 5 mM glutamine. +F and +P indicate complementation with rpoN derived from FRD1 and PAO1, respectively. (c) Expression of an aceA::lacZ transcripational fusion during a growth cycle in LB. ●, PAO1; ○, PAO1rpoN. (d) Expression of an rpoN::lacZ transcripational fusion during a growth cycle in LB. ●, PAO1; ○, FRD1.](http://mic.sgmjournals.org)
factor AlgT (AlgU), which is responsible for expression of alginate and hydrogen cyanide biosynthesis genes (Carterson et al., 2004; Wozniak & Ohman, 1994). While aceA expression does not appear to be under AlgT control, other factors may help to coordinate functions that impact chronic infection.

The correlation between ICL enzymic activity and aceA transcription in PAO1 has been noted by others (Kretzschmar et al., 2008). However, our results suggested that ICL activity is also regulated post-transcriptionally. As demonstrated in Fig. 2, heptanoic acid induced aceA::lacZ activity more strongly than palmitic acid induced the activity; however, the resulting ICL activity was higher following growth of PAO1 on palmitic acid compared with that on heptanoic acid. In the FRD1 background, ICL activity was clearly upregulated following growth on C2 carbon sources for which a corresponding increase in aceA::lacZ expression was lacking. ICL activity in other bacteria is allosterically affected by various metabolites (Hoyt et al., 1991; Kumar & Bhakuni, 2008; MacKintosh & Nimmo, 1988; Reinscheid et al., 1994). Currently, such information is lacking for P. aeruginosa ICL.

Our predictive approach failed to identify the mechanism of aceA deregulation in FRD1. However, an alternative approach identified that RpoN negatively regulates aceA expression and ICL activity in PAO1. In contrast, disruption of rpoN in FRD1 had no effect on aceA expression or ICL activity, even though introduction of rpoN from PAO1 on a multicopy plasmid reduced aceA expression in FRD1. The FRD1 rpoN appears to be functional, as indicated by complementation of a PAO1 rpoN mutant for ICL activity. In addition to FRD1, we also tested two pairs of isogenic sequential isolates [described by Smith et al. (2006)] that had either a functional or a mutated rpoN for ICL activity. As expected, both strains with wild-type rpoN showed low ICL activity that was similar to that of PAO1 (data not shown). However, only one of the rpoN mutant derivatives showed high ICL activity, further emphasizing the complexity of ICL regulation in CF isolates of P. aeruginosa.

No other complementing clones for aceA::lacZ expression were identified from the PAO1 genomic library using the alternative approach. This suggests that the library is incomplete, that the genetic screen was faulty, or that multiple mutations are required to cause deregulation of aceA in FRD1. We are continuing our study to identify regulators of aceA using other alternative approaches.

RpoN is widely distributed among bacteria and is involved in regulation of a variety of functions, including nitrogen assimilation, motility, virulence and carbon source utilization. In other pseudomonads, disruption of rpoN affects growth on several C2 dicarboxylic acids, such as succinate and fumarate (Hendrickson et al., 2000; Kohler et al., 1989), indicating a role in central metabolism. However, to our knowledge, our study is the first report of RpoN affecting transcription of aceA in any bacterium. In P. aeruginosa, RpoN plays a role in alginate biosynthesis (Kimbara & Chakrabarty, 1989), flagellin and pilin production (Totten et al., 1990), and production of several virulence determinants (Heurler et al., 2003). Indeed, RpoN is required for PA14 virulence on various hosts (Hendrickson et al., 2001). However, loss of RpoN may facilitate chronic colonization, as suggested by persistence of P. aeruginosa within the CF lung lacking this sigma factor (Smith et al., 2006). Benefits acquired by loss of RpoN include resistance to several antibiotics (Smith et al., 2006; Viducic et al., 2007), increased production of elastase and rhamnolipid (Heurler et al., 2003) and resistance to phage infection (Webb et al., 2003). Our data demonstrate that, although RpoN is active in the CF isolate FRD1, RpoN-mediated negative regulation of aceA is defective. Further study is required to decipher the mechanism by which RpoN negatively regulates aceA, and its role in chronic infection.

Induction of the glyoxylate pathway during chronic infection is common to several bacterial pathogens, including P. aeruginosa (Fang et al., 2005; McKinney et al., 2000; Son et al., 2007). However, the role of this pathway in chronic infection is poorly understood. Recently, van Schaik et al. (2009) have shown that ICL is a persistence factor for Burkholderia pseudomallei in pulmonary melioidosis. Unexpectedly, the B. pseudomallei mutants showed increased cytotoxicity towards macrophages, and inhibition of ICL activity forces B. pseudomallei away from the persistence state, and into a replicating state. Fortunately, the replicating state of B. pseudomallei is more susceptible to antibiotic therapy, and this raises the possibility of controlling these infections with ICL inhibitors. Our studies suggest that the glyoxylate pathway may be required for catabolism of certain nutrients by P. aeruginosa within the CF lung, and that it is required for optimal production of several virulence determinants. Bacterial metabolic pathways that are active during infection are attractive targets for therapeutic intervention if they are proven to be essential and non-redundant. A better understanding of the glyoxylate pathway and other metabolic pathways active in chronic isolates of P. aeruginosa is required before such targets become recognized.

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