Growth phenotypes of *Pseudomonas aeruginosa lasR* mutants adapted to the airways of cystic fibrosis patients

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Summary

The opportunistic pathogen *Pseudomonas aeruginosa* undergoes genetic change during chronic airway infection of cystic fibrosis (CF) patients. One common change is a mutation inactivating *lasR*, which encodes a transcriptional regulator that responds to a homoserine lactone signal to activate expression of acute virulence factors. Colonies of *lasR* mutants visibly accumulated the iridescent intercellular signal 4-hydroxy-2-heptylquinoline. Using this colony phenotype, we identified *P. aeruginosa lasR* mutants that emerged in the airway of a CF patient early during chronic infection, and during growth in the laboratory on a rich medium. The *lasR* loss-of-function mutations in these strains conferred a growth advantage with particular carbon and nitrogen sources, including amino acids, in part due to increased expression of the catabolic pathway regulator CbrB. This growth phenotype could contribute to selection of *lasR* mutants both on rich medium and within the CF airway, supporting a key role for bacterial metabolic adaptation during chronic infection. Inactivation of *lasR* also resulted in increased β-lactamase activity that increased tolerance to ceftazidime, a widely used β-lactam antibiotic. Loss of LasR function may represent a marker of an early stage in chronic infection of the CF airway with clinical implications for antibiotic resistance and disease progression.

Introduction

The bacterium *Pseudomonas aeruginosa* undergoes substantial genetic change during chronic infection of cystic fibrosis (CF) patient airways (Smith et al., 2006). Infants with CF are commonly infected shortly after birth, at least intermittently, with various bacterial species, including *P. aeruginosa* (Burns et al., 2001). Despite antibiotic treatment, *P. aeruginosa* ultimately reaches densities as high as 10⁶ cfu ml⁻¹ of sputum in most patients, and its persistence correlates with airway inflammation and progressive lung damage (Ramsey et al., 1999). As the *P. aeruginosa* population expands in the CF airway, it diversifies, yielding strains with traits not characteristic of environmental isolates. The same traits are consistently acquired by *P. aeruginosa* during chronic infection of different CF patients, suggesting that there is a conserved pattern of evolution by which this opportunistic pathogen adapts to the CF airway. Understanding the selection pressures driving this evolution is likely to be useful in the design of new treatment strategies directed at eradicating *P. aeruginosa*.

The phenotypes acquired by *P. aeruginosa* isolates over the course of CF airway infection reflect alterations in diverse aspects of their biology. These phenotypes can include lack of swimming motility (Mahenthiralingam et al., 1994); lack of expression of the Type III secretion system with an associated decrease in cytotoxicity (Jain et al., 2004); lack of production of several factors, including the siderophore pyoverdine (De Vos et al., 2001), O-antigen polysaccharide (Hancock et al., 1983; Spencer et al., 2003), and pyocins (Römling et al., 1994); increased phage resistance (Römling et al., 1994); structural alterations in the lipid A component of the outer membrane (Ernst et al., 2006); overproduction of alginate, the exopolysaccharide underlying the mucoid phenotype.
Deretic, 1996). Although these apparent adaptations are conserved, there remains extensive \textit{P. aeruginosa} phenotypic diversity, evident not only when comparing isolates from different CF patients, but even when comparing multiple isolates from the same CF respiratory specimen (Burns \textit{et al}, 2001; Smith \textit{et al}, 2006). Many factors could contribute to such bacterial diversity, including patient genotype (affecting airway physiology and immune response), antibiotic therapy, bacterial interspecies competition, and the duration of bacterial colonization.

Despite the complexity of \textit{P. aeruginosa} evolution in CF, comprehensively cataloguing the evolved phenotypes may reveal common themes, including sets of bacterial characteristics that distinguish stages in chronic airway infection. Uncovering such themes is made easier by the fact that CF patients commonly harbour clonal \textit{P. aeruginosa} lineages that persist for years, some starting with colonization shortly after birth. The evolution of these lineages is characterized by the accumulation of individual mutations. Thus, the set of acquired mutations in individual isolates reflects the natural history of genetic adaptation.

Sequential parentally related \textit{P. aeruginosa} isolates from one CF patient (designated CF Patient 1) sampled over the first 8 years of life have been analysed by DNA sequencing, including whole-genome sequencing of the earliest and latest isolate (Smith \textit{et al}, 2006). Compared with the earliest isolate, the latest isolate had accumulated 68 mutations, and intermediate isolates carried subsets of these mutations. Genetic analysis of \textit{P. aeruginosa} isolates from the airways of multiple CF patients revealed that one of the most common targets of mutation in CF isolates is the \textit{lasR} gene (Smith \textit{et al}, 2006). \textit{P. aeruginosa} \textit{lasR} mutants were found to emerge in 19 out of 30 CF patients, although in four of the nine cases where two isolates from one patient were examined, one of the isolates had a wild-type \textit{lasR} allele (Smith \textit{et al}, 2006). Most \textit{lasR} mutants carried mutations predicted to confer a loss of function, and in four cases the \textit{lasR} gene had acquired two mutations (Smith \textit{et al}, 2006). Together, these data suggest that there is strong selection pressure within the CF airway for loss of LasR function.

LasR is a transcriptional regulator that responds to the extracellular signal \textit{N}-(3-oxododecanoyl)-homoserine lactone synthesized by LasI (Schuster and Greenberg, 2006). The accumulation of the LasI-generated signal as a function of cell density is the basis for one of the two \textit{P. aeruginosa} quorum-sensing systems based on homoserine lactones, and the other system (composed of RhlRI) is itself regulated by LasR. LasR activates the expression of secreted factors, such as elastase (a tissue-degrading secreted protease), that play a role in acute virulence (Gambello and Iglewski, 1991). Inactivation of \textit{lasR} reduces \textit{P. aeruginosa} acute virulence in diverse model hosts, including plants, insects, nematode worms, protozoa, fungi and mice (D’Argenio, 2004). Consequently, selection in the CF airway for \textit{lasR} mutants could be due to selection against the expression of LasR-regulated acute virulence determinants. However, LasR also regulates a variety of genes involved in central metabolic functions in \textit{P. aeruginosa} (Schuster \textit{et al}, 2003; Wagner \textit{et al}, 2003; Heurlier \textit{et al}, 2006). Therefore, we investigated potential metabolic advantages conferred by inactivation of \textit{lasR}, and defined distinct \textit{in vitro} growth phenotypes that correlated with the acquisition of \textit{lasR} mutations during \textit{P. aeruginosa} genetic adaptation to the CF airway.

### Results

**Pseudomonas aeruginosa lasR mutants emerge early in CF airway infection and multiple alleles can occur even among strains of the same parental lineage**

To better understand the basis for selection of \textit{lasR} mutants in the airways of CF patients, we characterized sequential \textit{P. aeruginosa} isolates, including parental lineages in which \textit{lasR} mutants emerged. We initially focused on isolates sampled over the first 8 years of life of CF Patient 1, because the earliest and latest isolate had been analysed by whole-genome sequencing (Smith \textit{et al}, 2006). Compared with the earliest isolate (CF416), the latest isolate (CF5296) had accumulated 68 mutations (Smith \textit{et al}, 2006), one of which was a single nucleotide deletion in \textit{lasR} (Table 1). In addition, targeted DNA sequencing (Smith \textit{et al}, 2006) identified a missense mutation in \textit{lasR} in the intermediate isolate CF1323 (Table 1).

DNA sequencing of the entire \textit{lasR} gene in sequential isolates from CF Patient 1, as opposed to the partial sequencing performed previously to detect one allele (Smith \textit{et al}, 2006), identified four different \textit{lasR} mutations among these isolates (Table 1), indicating that \textit{lasR} mutants had emerged at least four times independently. The earliest \textit{lasR} mutant, CF1213, was isolated when the patient was 21 months of age; two isolates, each with a different \textit{lasR} mutation, were recovered from the patient at 24 months of age, one from the upper airway and one from the lower airway (Table 1). The coexistence in the airway of multiple \textit{P. aeruginosa} lineages carrying a \textit{lasR} mutation is consistent with strong selection pressure for loss of LasR activity. However, mutations in additional genes may determine the degree to which one \textit{P. aeruginosa} lineage can overgrow the bacterial population. The five isolates recovered from CF Patient 1...
between the ages of 5 and 8 years each carried the same single nucleotide deletion in lasR (Table 1), suggesting that one P. aeruginosa lasR mutant lineage had become established in the airway.

Mutations in lasR included point mutations as well as those caused by an insertion sequence (IS) element related to members of the IS4 (Table 1). Partial DNA sequence of this IS element was identical to that of one previously implicated in the loss of O-antigen biosynthesis and the loss of twitching motility (by inactivation of an O-antigen biosynthetic gene and the pilA gene respectively) in a subset of P. aeruginosa isolates from CF Patient 1 (Spencer et al., 2003; Smith et al., 2006). IS elements, such as this one, that actively transpose in the genome may contribute to faster evolution of P. aeruginosa lineages in individual CF patients (Kresse et al., 2006). A related element, ISPa1635, was identified as an insertion mutation inactivating oprD and associated with carbapenem resistance in a P. aeruginosa clinical isolate (Wolter et al., 2004).

### Inactivation of lasR confers on P. aeruginosa a growth advantage with amino acids

As part of a pioneering phenotypic characterization of members of the genus Pseudomonas, Stanier and colleagues tested a collection of 29 P. aeruginosa isolates (from environmental and clinical sources) for their ability to use each of 146 compounds as sole carbon source (Stanier et al., 1966). The ability to catabolize particular amino acids was not conserved; L-serine, for instance, was a good growth substrate for only four isolates, all cultured from the human airway (Stanier et al., 1966). Because the airway secretions of CF patients are known to be particularly rich in amino acids (Ohman and Chakrabarty, 1982; Barth and Pitt, 1996), we wondered whether genetic adaptation of P. aeruginosa to the CF airway might be reflected in enhanced ability to use amino acids as nutrients. We therefore tested sequential isolates from CF patients for their ability to catabolize individual amino acids. Among the 20 amino acids tested, growth with phenylalanine most clearly distinguished a subset of the isolates. In pairs of isolates from four CF patients (Patients 1, 3, 6 and 28), the isolate collected latest in infection, each carrying a lasR mutation (Smith et al., 2006), also displayed a growth advantage with phenylalanine when compared with the earliest (lasR wild-type) isolate. In each isolate pair, the lasR mutants reached a higher density after 48 h of growth with phenylalanine (Fig. 1A); in contrast, none of these mutants had a growth advantage with succinate as sole carbon source (data not shown) demonstrating that the

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**Table 1. DNA sequence of lasR in sequential isolates from CF patient 1.**

<table>
<thead>
<tr>
<th>Patient age (months)</th>
<th>Source(^a)</th>
<th>Isolate(^b)</th>
<th>Strain</th>
<th>lasR(^c)</th>
<th>LasR(^d)</th>
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<tr>
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<td>WT</td>
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<td>WT</td>
<td>WT</td>
<td>WT</td>
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<tr>
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<td>CF1328</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
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<td>OP E</td>
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<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
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<td>CF1326</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
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<td>WT</td>
<td>WT</td>
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<td>FU21</td>
<td>C147del Frameshift</td>
<td></td>
<td></td>
</tr>
<tr>
<td>96</td>
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<td>CF5295</td>
<td>C147del Frameshift</td>
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<td></td>
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<tr>
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<td>CF5296</td>
<td>C147del Frameshift</td>
<td></td>
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</tr>
</tbody>
</table>

\(a\). P. aeruginosa strains were isolated from the upper airway by a swab of the oropharynx (OP), from the lower airway by bronchoalveolar lavage (BAL), or from sputum.

\(b\). Bacterial isolates, each with a distinct morphotype, were ranked by prevalence in each sample, the most prevalent given the A designation (in each case, no more than 10-fold more numerous than other morphotypes); isolates from sputum were not ranked (NR). In some samples, P. aeruginosa was not the predominant organism (where the A or subsequent designations are absent).

\(c\). Numbering is based on the sequence of the lasR gene and the LasR protein from P. aeruginosa PAO1, and is preceded and followed by the parental and mutant sequence respectively. The target site duplication (2 IS insertion) was given in parentheses, the element inserted such that transcription of its transposase gene was oriented in the opposite (\(\sim\)) orientation relative to transcription of lasR, and partial DNA sequence of this element was identical to that annotated as a repeat region in GenBank accession number AF540990. The reference lasR sequence (WT) in CF416 is identical to that in PAO1.
growth advantage of lasR mutants is specific to particular carbon sources.

To determine whether inactivation of lasR was sufficient to generate the growth advantage with phenylalanine, we inserted a gentamicin-resistance gene in lasR in the earliest isolate from six CF patients (including Patients 1, 3 and 6). All six engineered lasR mutants had a growth advantage (Fig. 1A), demonstrating that inactivation of lasR enhanced catabolism of phenylalanine. Furthermore, this advantage extended to additional amino acids: the engineered mutant CF416lasR::Gm reached cell densities relative to the parental isolate CF416 that were 12-fold, threefold and twofold higher, respectively, after growth for 48 h with phenylalanine, isoleucine and tyrosine (Fig. 1B).

The growth advantage with phenylalanine displayed by lasR mutants could be at the level of transport or degradation. In either case, the antimetabolite fluorophenylalanine, a phenylalanine analogue that has long been used to study amino acid metabolism in P. aeruginosa (Fiske et al., 1983), would be predicted to differentially affect the growth of lasR mutants. Indeed, this was the case. In contrast to cultures of CF416, CF416lasR::Gm cultures incubated with succinate in the presence of fluorophenylalanine displayed a significant lag in growth, and the cfu in CF416lasR::Gm cultures remained at approximately the same level 24 h after inoculation (Fig. 1B). The two strains grew equally well with succinate alone (Fig. 1B).

To analyse the phenotypic consequences of a lasR mutation that had been acquired during chronic P. aeruginosa infection, we chose to characterize CF1213. As the earliest lasR mutant recovered from CF Patient 1 (Table 1), this isolate would be predicted to have
acquired mutations in fewer additional genes than later isolates, and thus to display phenotypes that better reflected loss of LasR activity. We restored the wild-type lasR gene in the chromosome of CF1213, generating strain CF1213R, and compared the growth of the two strains with phenylalanine. The lasR mutant CF1213 grew relatively rapidly to a cell density of 10^6 cfu ml^-1 before growth slowed, while the growth of CF1213R slowed after reaching a density of approximately 10^5 cfu ml^-1 (Fig. 1C), suggesting that the growth advantage of lasR mutants is due to derepressed growth at high cell density. Increasing the phenylalanine concentration from 0.1% (6 mM) to 0.5% (30 mM) did not eliminate the growth advantage (data not shown), indicating that the amount of phenylalanine in the culture was not growth limiting for either strain.

A lasR mutant of the P. aeruginosa laboratory strain PA14 also displayed the growth advantage (Fig. 1A); therefore, this phenotype is not specific to the genetic background of CF isolates. The one exception was the lasR mutant of the laboratory strain PAO1, which lacked a growth advantage with phenylalanine (Fig. 1A). The significance of this exception is unclear, however. It has been noted that the original PAO1 strain was lost (Heurlier et al., 2005), and that PAO1 strains from different laboratories are genotypically different (Maseda et al., 2000; Köhler et al., 2001) and vary in phenotypes that are dependent on quorum sensing (Köhler et al., 2001; Cosson et al., 2002).

Pseudomonas aeruginosa lasR mutant colonies can be identified by their metallic iridescent sheen due to accumulation of 4-hydroxy-2-heptylquinoline

The P. aeruginosa lasR mutants in this study were distinguished not only by their growth advantage with phenylalanine (Fig. 1A), but also by their distinctive colony morphology: a metallic iridescent sheen on the surface of colonies and lawns of cells after extended incubation on Luria–Bertani (LB) agar (Fig. 2A, data not shown). This P. aeruginosa phenotype, described at least as early as 1899 and rediscovered multiple times (Zierdt, 1971), has been linked to cell lysis (Hadley, 1924; Zierdt, 1971; D’Argenio et al., 2002). As demonstrated with the lasR mutant CF1213 (Table 1), the confluent lysed surface of colonies floated when the agar was flooded with liquid, and had a metallic iridescent sheen (Fig. 2B, Right). P. aeruginosa PA103, a strain isolated from sputum, and whose lack of LasR activity was used to first clone the lasR gene (Gambello and Iglewski, 1991), has also been noted to display iridescent autolysis (Whitchurch et al., 2005). This phenotype, although reproducibly manifested after extended incubation (Fig. 2A), may be masked in CF isolates with even more severe perturbations in colony morphology, including mucoidy and the small-colony morphtype (Häussler et al., 1999). Again, the lasR mutant derived from PAO1 was an exception, and lacked not only the growth advantage but also iridescent autolysis, both phenotypes that were displayed by the PA14 lasR mutant and all other lasR mutants tested (data not shown).

A biochemical analysis of iridescent material from P. aeruginosa cells not characterized at the genetic level (Wensinck et al., 1967) detected molecules that were predicted to be 4-hydroxy-2-alkylquinolines (HAQs). HAQs comprise a family of compounds at least two of which are used by P. aeruginosa in intercellular communication: the Pseudomonas quinolone signal (PQS) and its biosynthetic precursor 4-hydroxy-2-heptylquinoline (HHQ) (Pesci et al., 1999; Déziel et al., 2004; Xiao et al., 2006). Inactivation of lasR in the laboratory strain PA14 results in accumulation of HHQ (Déziel et al., 2004). This made HHQ a candidate for the cause of the iridescent metallic sheen of colonies of lasR mutants. To test this, we generated lawns of PA14 lasR mutant cells, and flooded them with liquid. Iridescent material floated to the surface (data not shown), as is the case for CF isolates (Fig. 2B), and extracts of this material (and of the cells themselves) were greatly enriched for HHQ compared with similar extracts derived from wild-type PA14 lawns (Fig. 2C). Addition of pure HHQ to a lawn of P. aeruginosa mutant cells lacking HAQs also generated an iridescent metallic sheen (Fig. 2D), while this phenotype was eliminated in a lawn of lasR mutant cells by treatment with methyl anthranilate, an inhibitor of HAQ biosynthesis (Calfee et al., 2001; Fig. 2E). Together, these data suggest that the iridescent metallic sheen associated with colonies of lasR mutants (Fig. 2A) is due to the iridescence of the HHQ signal itself, and that this phenotype can be used to identify lasR mutants.

Spontaneous lasR mutants emerge during growth on rich medium

During extended growth on LB agar, colonies of CF416, the earliest P. aeruginosa isolate from CF Patient 1, occasionally but reproducibly generated distinct sectors (Fig. 3A). The interiors of the largest sectors commonly were iridescent (Fig. 3B). Cells purified from a subset of the sectors displayed a set of phenotypes that strikingly overlapped with those of the lasR mutants CF416lasR::Gm and CF1213. These spontaneous mutants, typified by strain CF416L1, formed colonies identical in appearance to CF416lasR::Gm (Fig. 3C), sharing not only large colony size, but also autolysis (Fig. 2B) and a metallic sheen (data not shown); CF416L1 cells also had a growth advantage with phenylalanine (Fig. 1C).

Given all of these similarities, we sequenced the lasR gene in CF416L1, revealing the same IS element insertion as that in two of the isolates from CF Patient 1
Fig. 2. Inactivation of lasR results in visible accumulation of HHQ.

A. Growth on LB agar, photographed after incubation overnight at 37°C followed by 7 days at 22°C (strain names are shown schematically above). The surface of the six lasR mutant colonies has a metallic sheen.

B. Colonies of three strains growing on LB agar, photographed after incubation overnight at 37°C followed by 3 days at 22°C, before (Left) and after (Right) flooding with crystal violet stain (0.04%) covered and obscured CF416, and floated the lysed surface of colonies of the lasR mutants CF416L1 and CF1213. White bar = 5 mm.

C. Analysis of HAQs extracted from two fractions of a lawn of cells of PA14 and PA14/lasR::Gm, one released by flooding the lawn with liquid (floating material), and one by subsequently suspending the cells themselves (agar surface).

D. HHQ (100 μg in 10 μl methanol) confers a metallic sheen (arrow) to a lawn of cells of a pqsApqsH mutant lacking HAQs and incapable of converting HHQ to PQS. Methanol alone had no effect (data not shown).

E. Methyl anthranilate fumes generated a circular zone (arrow) in which the iridescent metallic sheen in a lawn of cells of CF416/lasR::Gm was suppressed. Methyl anthranilate was added to a filter disk on the lid of an inverted Petri dish, and the agar surface was photographed after incubation overnight at 37°C followed by 3 days at 22°C.

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(Table 1), except that the element was in the opposite orientation (Table 2). Although such lasR mutants can arise during growth in the laboratory, the distinctive colony morphology of the lasR mutants isolated from CF Patient 1 was identified at the time of isolation (Burns et al., 2001), arguing that the lasR mutations in these isolates were acquired in vivo. Restoring the wild-type lasR gene in the chromosome of CF416L1, generating strain CF416L1R, restored wild-type colony morphology (Fig. 3C) and eliminated the growth advantage with phenylalanine (data not shown), just as with strain CF1213R (Figs 1C and 3C). Therefore, the lasR mutation is necessary for the colony morphology and growth advantage of the lasR mutants that emerge either in vitro or in vivo.

Spontaneous lasR mutants emerged as discrete sectors that grew beyond the borders of individual colonies (Fig. 3B). This was not because the parental strains were defective in twitching motility, because both the parental and derived strains were proficient at this form of surface spreading (Fig. 3D, Lower). Furthermore, colony sectors composed of lasR mutants expanded into areas between dense bacterial populations, a zone where the spreading of parental cells was visibly inhibited (Fig. 3A).

Such uninhibited growth suggests parallels with the derepressed growth at high cell density observed for lasR mutants growing with phenylalanine (Fig. 1C). We hypothesize that derepressed growth is selected on LB agar, a medium rich in nutrients (including amino acids), selection pressure that might not typically exist in the more nutrient-poor soil and water environments in which P. aeruginosa can be found.

Although lysis of a fraction of the bacterial population is a potential cost associated with loss of LasR activity (Fig. 2B), the importance of this cost may vary depending on growth conditions. Autolysis was clearly affected by growth medium: during growth of CF416L1 on brain heart infusion agar, autolysis was visible as a distinct zone of clearing within the colony (Fig. 3D, Upper), rather than the confluent lysis seen during growth with LB medium (Fig. 2B). In addition, autolysis of lasR mutants can be suppressed by the presence of wild-type cells. When cells of CF416 were grown on a lawn of CF416L1, the metallic sheen and lysis of CF416L1 was suppressed in a zone surrounding CF416 (Fig. 3E), suggesting that a metabolite diffusing from wild-type cells could complement these phenotypes. Such complementation could ameliorate the
The lasR alleles in spontaneous mutants that emerge in vitro are shared by lasR mutants that emerge in vivo

Colony sectoring during growth on LB agar was characteristic of P. aeruginosa strains from diverse sources. We examined CF isolates (CF416 as well as strain 3-0.8, the earliest P. aeruginosa isolate from another young CF patient), an environmental isolate (strain ENV25 from a supermarket vegetable), and the laboratory strain PA14. In all four of these cases, colony sectoring was apparent, and a subset of the sectors was composed of cells that grew into colonies resembling those of lasR mutants (Figs 2A and 3C). Given its prevalence, colony sectoring on LB agar was unlikely to be due to hypermutability, a phenotype that can emerge in CF isolates as a result of mutations that inactivate DNA mismatch repair enzymes (Oliver et al., 2000), and that can be detected by an increased yield of spontaneous rifampicin-resistant or streptomycin-resistant mutants (Oliver et al., 2000; Maciá et al., 2005). Indeed, growth of CF416 generated an equivalent number of spontaneous rifampicin-resistant mutants as growth of the non-hypermutable laboratory strain PAO1 (an average of 1 per 10^6 cells), and the yield of streptomycin-resistant mutants was also equivalent in the two strains (data not shown).

As spontaneous mutants that shared phenotypes with CF416L1 were so readily obtainable from sectors in colonies grown on LB agar, we wondered whether they all carried a mutation in lasR. We analysed 29 such mutants, 14 derived from strain CF416 (including CF416L10, isolated from the sector visible in Fig. 3A, and CF416L1), 13 from strain 3-0.8, one from strain ENV25, and one from strain PA14. To prevent the isolation of siblings, only one sector was sampled from each colony. All 29 of the variants carried a mutation altering the lasR gene relative to the parental strain, demonstrating that spontaneous lasR mutants arising in vitro can be identified by their colony morphology as readily as those from CF patients. The absence of spontaneous lasI mutants was striking, given that an engineered lasI mutant derived from CF416 had the same colony morphology and growth advantage with phenylalanine as CF416LasR::Gm (data not shown). This preferential recovery of lasR mutants is likely to reflect the fact that, for single P. aeruginosa cells within a colony, the potential advantages associated with a spontaneous mutation inactivating lasR are immediate (because of elimination of the LasR receptor for the homoserine lactone signal), while the phenotypes of spontaneous mutations inactivating lasI will be complemented by LasI-generated signal produced from neighbouring wild-type cells in the colony.

We identified a mutation confined to the lasR gene in 26 of the 29 spontaneous mutants (Table 2). Of the three remaining mutants (derived from strain 3-0.8), one carried a 1425 bp deletion extending from within lasR into the adjacent gene PA1429, and the lasR gene in two mutants was not amplified by PCR, presumably because of a DNA deletion or rearrangement. The majority of spontaneous lasR mutations were insertions, deletions or nonsense mutations (Table 2). Missense mutations, although a minority, affected amino acid residues predicted to be important for LasR function based on amino acid conservation of LasR with other members of the LuxR family of regulators, and on structural data for the LuxR-type regulator TraR (Chugani et al., 2001; Vannini et al., 2002; Zhang et al., 2002): LasR residues Y64 and D73 are predicted to directly interact with the homoserine lactone signal, while residue R216 is predicted to interact with DNA (Fig. 4).

Several spontaneous lasR mutants carried the same mutation (Table 2). In six of the spontaneous mutants derived from CF416, including CF416L10 (Fig. 3A), an IS element had inserted in the same site in lasR in either of

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Table 2. Mutations in lasR in spontaneous mutants that emerged during in vitro growth.

<table>
<thead>
<tr>
<th>lasR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LasR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Strain&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>L154F</td>
<td>CF171BL11</td>
</tr>
<tr>
<td>2&gt;(G463–464)</td>
<td>Frameshift</td>
<td>CF146L16</td>
</tr>
<tr>
<td>C473A</td>
<td>A158E</td>
<td>CF146L19</td>
</tr>
<tr>
<td>2&gt;(512–518)</td>
<td>Frameshift</td>
<td>CF146L12</td>
</tr>
<tr>
<td>2&gt;(552–561)::IS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>IS insertion</td>
<td>CF146L1, 4, 18</td>
</tr>
<tr>
<td>2&gt;(552–561)::IS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>IS insertion</td>
<td>CF146L2, 3, 10</td>
</tr>
<tr>
<td>G571T</td>
<td>G191C</td>
<td>CF146L6, PA14L1</td>
</tr>
<tr>
<td>C646T</td>
<td>R216W</td>
<td>CF171BL7</td>
</tr>
</tbody>
</table>

a. Numbering is based on the sequence of the lasR gene and the LasR protein from P. aeruginosa PAO1, and is preceded and followed by the parental and mutant sequence respectively. Duplicated DNA sequences (2>) are given in parentheses, and include the 10 bp target site duplication (5′-GTGGCATGG-3′ in one case) of an IS element (related to IS4 family members) in strain CF416-derived mutants, and the 3 bp target site duplication of an IS element (related to IS3 family members) in strain 3-0.8-derived mutants. The IS elements inserted such that transcription of their transposase gene was oriented in the same (+) or opposite (-) orientation relative to transcription of lasR.

b. Mutants with strain designations 416, 171B, ENV and PA14 are derived from strains CF416, 3-0.8, ENV25 and PA14 respectively.

The costs of lasR mutation in mixed P. aeruginosa populations, including those in the airways of young CF patients (Table 1).

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two orientations (Table 2), defining an insertion hot spot that is also a target of mutation in lasR mutants that emerged in vivo (Table 1). The nonsense mutation (G179A) identified in two spontaneous mutants of the CF isolate 3-0.8 (Table 2) was also acquired during evolution in the airway, because it was identified in strain 3-7.3 (Smith et al., 2006), which was isolated 6.5 years later than strain 3-0.8 from the same patient. The missense mutation G571T was identified in two spontaneous mutants of the CF isolate 3-0.8 (Table 2) was also acquired during evolution (Chugani et al., 2002; Zhang et al., 2002). Shown for comparison are the two residues (A67 and P117) altered in strains CF1323 and CF1213 (Table 1) respectively.

Fig. 4. LasR amino acid residues altered by spontaneous missense mutations. The LasR domain organization is shown, and is based on comparison with TraR; delineated below is the extent of the primary dimerization domain and the DNA recognition helix, shown above are the wild-type amino acid residues altered in spontaneous mutants, those in bold being conserved in the LuxR-homologues LasR, RhlR and OsrC from P. aeruginosa, and TraR from Agrobacterium tumefaciens (Chugani et al., 2001; Vannini et al., 2002; Zhang et al., 2002). Shown for comparison are the two residues (A67 and P117) altered in strains CF1323 and CF1213 (Table 1) respectively.

The growth advantage of lasR mutants that emerge in vitro is shared by those that emerge in vivo, and extends to growth with succinate/lactamide medium

All of the spontaneous lasR mutants that emerged in vitro (Table 2), including ENV25L1 derived from an environmental isolate, displayed a growth advantage with phenylalanine (Fig. 1A, data not shown). To characterize the growth advantage of lasR mutants in more detail, we used phenotype microarray (PM) analysis (Bochner, 2003) to compare the parental strain CF416 with the spontaneous lasR mutant CF416L1. Inactivation of lasR in CF416L1 resulted in a strikingly broad metabolic gain of function consisting of enhanced use of diverse compounds as sources of carbon, nitrogen, phosphorus or sulphur (Table S1). This growth advantage included enhanced use of five amino acids as carbon sources (alanine, asparagine, glutamine, histidine and proline) and nine amino acids as nitrogen sources (alanine, leucine, lysine, threonine, tryptophan, tyrosine and valine), with only alanine in common to both groups. A growth advantage with phenylalanine was not observed in this analysis for CF416L1, likely as a result of the higher inoculum cell density and shorter duration of the PM experiment compared with the experiment shown in Fig. 1C. Nevertheless, standard growth tests confirmed that inactivation of lasR in two different genetic backgrounds conferred a growth advantage with alanine as sole source of carbon and nitrogen (Fig. 5A).

Besides phenotypes with amino acids, CF416L1 displayed (relative to CF416) enhanced usage of ammonia, nitrate, nitrite, and a variety of dipeptides as nitrogen sources (Table S1). In addition, a PM analysis of nitrogen source usage by the lasR mutant CF1642, an isolate from CF Patient 1 at 3 years of age (Table 1), revealed a growth advantage with the same nine amino acids as CF416L1 (data not shown). Given that PM analysis of lasR mutants revealed conserved phenotypes related to nitrogen metabolism, we used a simple growth assay to test lasR mutants for the ability to use lactamide as sole nitrogen source during growth with succinate. It has been noted that such growth conditions can distinguish sequential isolates from CF patients (Silo-Suh et al., 2005). Inactivation of lasR in two different genetic backgrounds conferred an increased growth yield in succinate/lactamide medium (Fig. 5B). In addition, the lasR mutant that emerged in vitro, CF416L1, and two that emerged in vivo, CF1642 and CF5296 (Table 1), displayed growth in succinate/lactamide medium that appeared to be derepressed at high cell density (Fig. 5C) in a manner conspicuously similar to that observed during growth with phenylalanine (Fig. 1C).

Inactivation of lasR confers alterations in transcriptional profile that are conserved in isolates from multiple CF patients

Loss of the LasRI quorum-sensing system has been shown to lead to extensive changes in the transcriptional profile of P. aeruginosa cells (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003; Salunkhe et al., 2005). To define the set of transcriptional changes associated with inactivation of lasR in isolates from CF patients, we used DNA microarray analysis of P. aeruginosa cells grown with LB medium to stationary phase. We compared the transcriptional profiles of P. aeruginosa isolates collected from three different CF patients early in infection with the profiles of parentally related lasR mutants that emerged later in infection. All of the lasR mutations that arose in vivo in these lineages are predicted to be null mutations: a 1 bp deletion in strains CF5296 and 6-9.6B, and a nonsense mutation in strain 3-7.3 (Smith et al., 2006). Because the genome sequence...
of CF5296 (the isolate that was derived from CF416 by evolution in the airway of CF Patient 1) revealed that it had accumulated 68 mutations (Smith et al., 2006), we also compared CF416 with CF416 lasR::Gm in the transcriptional analysis so as to identify the set of changes due specifically to loss of LasR activity.

Inactivation of lasR correlated with conserved alterations in transcriptional profile (Table S2), and the genes with decreased expression in the lasR mutants in all four comparisons included 24 of the 76 genes in common among three analyses of the quorum-sensing regulon in strain PAO1 (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003). Particularly apparent was that mutation of lasR in the CF isolates correlated with downregulated expression of rhlR and qscR, both of which encode LasR homologues that regulate distinct sets of genes in

![Fig. 5. Inactivation of lasR confers enhanced utilization of nitrogen sources that is partly dependent on the catabolic regulator CbrB.

A. Growth yields with alanine as sole source of carbon and nitrogen (ALA[C-N]) after 24 h of growth. Values are the average cfu of two to three cultures, and error bars show standard deviation. Cultures were inoculated with approximately 2 x 10^7 cfu ml^-1 of cells grown with succinate.

B. Growth yields with succinate/lactamide (SUC/LACT) after 24 h of growth. Values are the average cfu of three cultures, and error bars show standard deviation. Cultures were inoculated with approximately 2 x 10^7 cfu ml^-1 of cells grown with succinate.

C. Growth advantage with succinate/lactamide (SUC/LACT) medium displayed by the lasR mutant strains CF416L1, CF1642 and CF5296 as compared with the parental strain CF416. Cultures were inoculated with cells grown with succinate. Results are representative of at least three independent experiments.

D. Real-time PCR analysis of transcription of the cbrB gene in the lasR mutants CF5296 and CF416 lasR::Gm as compared with the parental strain CF416. Values are the averages of two independent experiments, and error bars show standard deviation.

E. Growth yields with succinate/lactamide (SUC/LACT) after 24 h of growth of strains with wild-type lasR carrying a plasmid expressing cbrB (pCbrB) or the empty vector (pVect). Values are the average cfu of three cultures, and error bars show standard deviation. Cultures were inoculated with approximately 2 x 10^7 cfu ml^-1 of cells grown on LB agar. Plasmids were maintained in liquid cultures with 50 μg ml^-1 gentamicin (for isolate 3-0.8) or 150 μg ml^-1 carbenicillin (for isolate 6-1).]
response to cell-to-cell signalling via homoserine lactones (Fuqua, 2006; Lequette et al., 2006). Although this result is consistent with previous studies showing that LasR is at the top of the quorum-sensing regulatory hierarchy (Fuqua, 2006; Lequette et al., 2006), the qscR gene had not previously been included in the LasR regulon, perhaps because of unique features of strain PAO1. Most importantly, the conservation of transcriptional profiles in the CF isolates (Table S2) indicates that the effects of loss of transcriptional activation by LasR can persist, and are not quickly reversed by subsequent mutations during evolution in the CF airway.

**Increased expression of the regulatory protein CbrB contributes to the growth advantage acquired by P. aeruginosa during genetic adaptation to the CF airway**

To search for the molecular basis of the growth advantage of lasR mutants, we performed a quantitative proteomic analysis with isotope-coded affinity tag (ICAT) reagents, a technique that we had used previously to characterize *P. aeruginosa* physiology during anaerobic growth (Wu et al., 2005). We grew bacterial cells with LB medium to early stationary phase (as in the transcriptional analysis), and compared the proteome of CF416 with the proteomes of CF5296 and CF416lasR::Gm. The expression of 120 proteins was altered in both comparisons, suggesting that these alterations were due to inactivation of lasR (Table S3).

The expression of 27 proteins was completely lost in the two lasR mutants, and included enzymes whose expression is known to be activated by LasR, such as enzymes for biosynthesis of phenazines (PhzB2, PhzC2 and PhzE2) and cyanide (HcnB and HcnC) (Table S3). Conversely, the expression of 13 proteins was increased at least threefold in both of the lasR mutants (Table 3), suggesting that inactivation of lasR, directly or indirectly, led to the increased expression of these proteins. This latter set included CbrB and AmiE. The increased level of CbrB protein was at least partially due to effects at the level of transcription, because we detected increased transcription of the cbrB gene in the lasR mutants CF5296 and CF416lasR::Gm (Fig. 5D).

CbrB is the histidine kinase member of the CbrAB two-component regulatory system, which is broadly involved in carbon and nitrogen catabolism (Nishijyo et al., 2001). Inactivation of CbrB impairs growth with diverse compounds as sole carbon source, including alanine, arginine, histidine, proline, ornithine, polyamines, mannitol, glucose, pyruvate, citrate and isocitrate (Nishijyo et al., 2001). AmiE is an aliphatic amidase one of whose substrates is lactamide (Brammar et al., 2001). The results of the proteomic analysis raised the possibility that loss of LasR activity results in increased expression of CbrB which in turn leads to increased expression of AmiE, thereby explaining the enhanced growth of lasR mutants with lactamide as nitrogen source (Fig. 5B and C). Consistent with this model, increasing the level of CbrB (by expression of cbrB on a plasmid from its own promoter) in the earliest isolates from two CF patients, each carrying a wild-type lasR gene on the chromosome (Smith et al., 2006), resulted in enhanced growth with lactamide as sole nitrogen source (Fig. 5E). Conversely, inactivation of cbrB in the lasR mutant CF416L1 (generating strain CF416L1cbrB::Gm) eliminated the growth advantage in succinate/lactamide medium (Fig. 5B), while not affecting growth with succinate alone (data not shown). Overexpression of cbrB did not confer enhanced growth with phenylalanine as sole carbon source (data not shown), suggesting that it could confer only part of the growth advantage of lasR mutants. We did not use the *P. aeruginosa* lineage from CF Patient 1 for these experi-

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**Table 3. Proteins increased in expression in lasR mutants (greater than threefold in both comparisons).**

<table>
<thead>
<tr>
<th>Gene number</th>
<th>Gene product</th>
<th>CF5296/CF416*</th>
<th>CF416lasR::Gm/CF416*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA0510b</td>
<td>NirE</td>
<td>14.29</td>
<td>≥ 100</td>
</tr>
<tr>
<td>PA1913</td>
<td></td>
<td>≥ 100</td>
<td>≥ 100</td>
</tr>
<tr>
<td>PA2112b</td>
<td></td>
<td>4.55</td>
<td>≥ 100</td>
</tr>
<tr>
<td>PA2625</td>
<td></td>
<td>6.25</td>
<td>3.70</td>
</tr>
<tr>
<td>PA3166</td>
<td></td>
<td>3.23</td>
<td>3.13</td>
</tr>
<tr>
<td>PA3366</td>
<td>AmiE</td>
<td>≥ 100</td>
<td>≥ 100</td>
</tr>
<tr>
<td>PA3392</td>
<td>NosZ</td>
<td>≥ 100</td>
<td>≥ 100</td>
</tr>
<tr>
<td>PA3976</td>
<td>ThiE</td>
<td>5.26</td>
<td>12.50</td>
</tr>
<tr>
<td>PA4329</td>
<td>PykA</td>
<td>≥ 100</td>
<td>7.69</td>
</tr>
<tr>
<td>PA4442b</td>
<td>CysN</td>
<td>≥ 100</td>
<td>≥ 100</td>
</tr>
<tr>
<td>PA4486b</td>
<td></td>
<td>≥ 100</td>
<td>7.14</td>
</tr>
<tr>
<td>PA4726</td>
<td>CbrB</td>
<td>4.17</td>
<td>6.25</td>
</tr>
<tr>
<td>PA5435</td>
<td></td>
<td>5.00</td>
<td>4.55</td>
</tr>
</tbody>
</table>

a. Values shown are a subset of the data in Table S3, and represent fold-increase in expression in the lasR mutant relative to CF416; values of ≥ 100 indicate that the protein was too low in abundance to be detected in CF416.

b. Proteins that were detected as upregulated in the proteome of isolate 5-9.6B, a CF isolate that did not acquire a lasR mutation (see Table S4); fold-increases were ≥ 100-fold (NirE), 3.21-fold (PA2112), 2.07-fold (CysN) and 2.26-fold (PA4486).
ments because of difficulties in maintaining plasmids in these isolates.

The proteomic analysis (Table S3), like the transcriptional (Table S2) and PM (Table S1) analyses, suggested that diverse metabolic pathways were altered due to inactivation of lasR. To determine if any of these alterations were also present in P. aeruginosa CF isolates that had not acquired a lasR mutation, we compared the proteome of isolate 5-2.3 with that of 5-9.6B (Table S4), the isolate that was derived from 5-2.3 by evolution in the airway of CF Patient 5, and that had maintained the same lasR allele (Smith et al., 2006). Of the 13 proteins whose expression appeared to be upregulated greater than threefold in lasR mutant CF5296, four proteins were also upregulated in 5-9.6B (Table 3), including NirE, involved in denitrification (Kawasaki et al., 1997), and CysN, involved in sulphate assimilation (Hummerjohann et al., 1998). This adds evidence that central metabolic pathways are the targets of selection during P. aeruginosa adaptation to the CF airway. We detected unaltered CbrB protein levels in the proteomic comparison of isolates 5-2.3 and 5-9.6B. Although LasR appeared to be slightly downregulated in 5-9.6B, proteins encoded by genes whose expression is activated by LasR (including hcn genes involved in cyanide biosynthesis and pqs genes involved in HAQ biosynthesis) were upregulated (Table S4). Thus, elements of the quorum-sensing regulon can be affected in opposite ways in P. aeruginosa lineages from different CF patients, and in the case of isolate 5-9.6B, such alteration is caused by a mutation in a gene other than lasR.

Inactivation of lasR results in increased β-lactamase activity and decreased killing by the β-lactam ceftazidime

Although the growth advantage with amino acids displayed by lasR mutants could contribute to their selection in the CF airway, such an advantage might be offset if lasR mutants had increased susceptibility to the antibiotics used to treat CF patients. In particular, given that lasR mutants (such as CF416L1) displayed autolysis (Fig. 2B), we wondered whether they were relatively susceptible to the β-lactam antibiotic ceftazidime. Ceftazidime, routinely used in the treatment of CF patients (Gibson et al., 2003), binds to penicillin binding proteins in P. aeruginosa, disrupting peptidoglycan biosynthesis and triggering cell lysis (Hayes and Orr, 1983).

Based on standard tests for broth minimum inhibitory concentration (MIC), loss of LasR activity did not enhance susceptibility to ceftazidime: strains CF416L1 and CF416 were equally susceptible to ceftazidime, and the MIC was 1.25 μg ml⁻¹ for both strains. In addition, spontaneous resistant mutants derived from both strains were readily selected by growth in the presence of ceftazidime (Fig. 6A). However, lawns of CF416L1 cells were unique in that small colonies arose (Fig. 6A) that were composed of cells shown subsequently not to be resistant to the original ceftazidime concentration in the agar. This suggested that the antibiotic in the agar was being partially inactivated by one of the β-lactamases encoded in the P. aeruginosa genome (Salunkhe et al., 2005). Indeed, the emergence of small colonies was inhibited by addition of tazobactam (Fig. 6A), a β-lactamase inhibitor (Giwerzman et al., 1990).

Assays with nitrocefin, a chromogenic indicator of β-lactamase activity (O’Callaghan et al., 1972), revealed that inactivation of lasR in nine different P. aeruginosa genetic backgrounds, including the laboratory strains PA14 and PAO1, resulted in increased β-lactamase activity in cell-free culture supernatants (Fig. 6B). In cultures of CF416lasR::Gm, the activity that was cell-associated (comprising the majority of the activity) combined with that in the supernatant was threefold greater than in the parental strain CF416 (Fig. 6C). Although this β-lactamase activity was insufficient to alter the MIC of ceftazidime during broth culture, it resulted in fewer cells of the lasR mutant CF416L1 being killed by ceftazidime at a wide range of concentrations (Fig. 6D). This tolerance was eliminated by restoration of a wild-type lasR gene in CF416L1R (Fig. 6D). These results suggest that β-lactam antibiotics could provide additional selection pressure favouring the emergence of lasR mutants in the CF airway. The expression of either ampC nor poxB, genes encoding known β-lactamases (Kong et al., 2005a, b), was increased in lasR mutants (Table S2), despite the increased β-lactamase activity of such mutants (Fig. 6B). A link between LasR and β-lactamase activity, however, is consistent with the fact that AmpR, a regulator of β-lactamase expression in P. aeruginosa, appears to be physiologically linked to the LasR regulon (Kong et al., 2005b).

Discussion

Pseudomonas aeruginosa phenotypic and genotypic diversity

Pseudomonas aeruginosa lasR mutants have been identified by DNA sequencing among isolates from diverse sources, particularly among sequential isolates from CF patients, indicating that lasR mutations can be acquired during growth in the CF airway (Cabrol et al., 2003; Dénervaud et al., 2004; Heurlier et al., 2005; Salunkhe et al., 2005; Smith et al., 2006). We found that prolonged incubation of diverse P. aeruginosa isolates (including CF isolates, an environmental isolate, and the laboratory reference strain PA14) resulted in the emergence on LB agar of colony sectors (Fig. 3A and B) composed of spontane-
ous lasR mutants. Spontaneous lasR mutants have also been found to emerge during extended growth with a rich medium in liquid culture, using either the laboratory strain PAO1 (Heurlier et al., 2005) or an environmental isolate (Luján et al., 2007). Thus, P. aeruginosa has a tendency to accumulate lasR mutants during growth in the CF airway and during in vitro growth with a rich medium. Such mutants commonly display a set of phenotypes that includes a distinctive colony morphology (Fig. 2A; Luján et al., 2007). P. aeruginosa isolates not characterized by DNA sequencing, especially spontaneous mutants that emerge in vitro, may carry a lasR mutation whenever their phenotypes include absence of the LasB protease (elastase) or reduced total protease, absence of the LasI-generated signal, the ability to grow well with phenylalanine, or large colony size together with colony iridescence.

Fig. 6. Inactivation of lasR confers increased β-lactamase activity and β-lactam tolerance.
A. Ceftazidime-resistant colonies emerged in a lawn of CF416 or CF416L1 cells on LB agar with 20 μg ml⁻¹ of ceftazidime (Petri dish upper and lower halves), but only the lasR mutant lawns yielded small partially resistant colonies whose growth is inhibited by the addition of 8 μg ml⁻¹ of the β-lactamase inhibitor tazobactam (Petri dish lower halves). Photographs were taken after incubation overnight at 37°C followed by 3 days at 22°C.
B. β-Lactamase activity in culture supernatants, measured as a change in optical density at 490 nm (ΔAbs) after addition of the chromogenic substrate nitrocefin. Cases with no change are marked with an asterisk. Values are the average of three technical replicates, and error bars show standard deviations. Equivalent results were obtained using a qualitative whole-culture assay.
C. β-Lactamase activity in whole cultures of CF416 and CF416LasR::Gm. Values are the averages of three cultures, and error bars show standard error of the mean. One unit of β-lactamase is defined as 1 μmol of nitrocefin hydrolysed per min per mg of total protein.
D. Reduced killing by ceftazidime of the lasR mutant CF416L1 relative to CF416 and CF416L1R. Values are the average cfu of two cultures, and error bars show standard deviations.
and metallic sheen (Hadley, 1924; Wahba, 1964; Zierdt and Schmidt, 1964; Stanier et al., 1966; Janda et al., 1982; Sheehan et al., 1982; Häussler et al., 1999; Schaber et al., 2004; Zhu et al., 2004; Hoffmann et al., 2005; Lee et al., 2005; Pirmay et al., 2005; Luján et al., 2007).

A lasR mutant of the laboratory strain PAO1 did not display autolysis, iridescent sheen, or a growth advantage with phenylalanine, all phenotypes that were conferred by inactivation of lasR in nine other genetic backgrounds in this study, including the laboratory strain PA14. Nevertheless, spontaneous lasR mutants emerged during extended growth of PAO1 in a medium rich in amino acids, an observation interpreted to be due to reduced lysis of the mutants (Heurlier et al., 2005). It is possible that the physiological advantage associated with inactivation of lasR is expressed to a lesser degree in PAO1, perhaps as a subtle catabolic advantage that enhances cell viability.

During serial passage of laboratory strains, they may acquire mutations that reflect adaptation to growth at 37°C with rich medium in single-species cultures, including the loss of traits that were shaped over evolutionary time by growth in complex microbial communities in the soil and water. The sample of PAO1 used for genome sequencing has lost twitching motility, in contrast to other strains of PAO1, and some strains of PAO1 have even acquired a lasR mutation (Heurlier et al., 2005). Given that lasR mutants so readily emerge during in vitro growth, it remains possible that some of these mutants, such as those identified among environmental isolates, acquired the mutation during or after isolation. Furthermore, individual strains and isolates of P. aeruginosa may inevitably fail to display all the phenotypes characteristic of the species. The laboratory strain PAK, for instance, is deficient in HAQ production, and does not make PQS (Lépine et al., 2003).

A primary role for LasR in P. aeruginosa metabolism

We were able to use the distinctive colony morphology of spontaneous lasR mutants to visually identify colonies of P. aeruginosa cells with lasR mutations in four different genetic backgrounds. This colony phenotype includes the lysis of a fraction of the cells, at least during growth on a rich agar medium (Figs 2B and 3D). While this indicates that loss of LasR activity can result in severe physiological perturbations, lasR mutants growing under other conditions, such as with phenylalanine as sole carbon source, have a clear advantage and more rapidly reach a higher population density (Fig. 1C). Furthermore, at the time of emergence of lasR mutant cells within a population of wild-type cells, autolysis may be restricted due to extracellular complementation (Fig. 3E). While autolysis of lasR mutants can vary depending on growth conditions, this phenotype adds evidence for a central role of LasR in P. aeruginosa metabolism.

Autolysis in lasR mutants can be suppressed by metabolites diffusing from wild-type cells (Fig. 3E), suggesting that a balance of metabolites in wild-type cells prevents autolysis. Indeed, such a possibility is supported by observations made with the laboratory strain PAO1. The pqsL gene is required for biosynthesis of a subset of HAQs (Lépine et al., 2004), and inactivation of pqsL results in autolysis, detected in strain PAO1 as iridescent zones of clearing in colonies (D’Argenio et al., 2002), and as liberated chromosomal DNA in liquid cultures (Allesen-Holm et al., 2006). Autolysis in pqsL mutants is suppressed by mutations that eliminate all HAQ production (D’Argenio et al., 2002; Déziel et al., 2004). The pqsL gene is LasR regulated: pqsL expression was downregulated in the four lasR mutants that we analysed with DNA microarrays (Table S2). Thus, a LasR-regulated balance of HAQs may limit autolysis in wild-type cells. The quorum-sensing controlled balance of HAQs may exert its effects in part at the level of the electron transport chain, given the properties of some of the extracellular products whose expression is under LasR control: the HAQ 4-hydroxy-2-heptylquinolone N-oxide is an analogue of quinone electron shuttles (Häse et al., 2001), cyanide is a cytochrome oxidase inhibitor (Blumer and Haas, 2000; Cooper et al., 2003), and pyocyanin can serve as an accessory respiratory electron acceptor (Friedheim, 1931; Price-Whelan et al., 2006). All of these products are commonly considered secondary metabolites, and further studies will be required to determine to what extent they play a central metabolic role in P. aeruginosa (Price-Whelan et al., 2006).

Regulatory loss that confers a catabolic advantage

Pseudomonas aeruginosa lasR mutants emerge during growth both in vitro and in vivo. One potential benefit acquired by such mutants is that they avoid the metabolic burden of producing exoproducts whose expression is LasR-dependent (Haas, 2006). However, this is unlikely to completely account for their selection: lasR mutants do not have a general growth advantage, rather they have an advantage with specific carbon and nitrogen sources, including particular amino acids (Table S1). We propose that P. aeruginosa isolates from the environment, upon infecting the airways of CF patients, undergo some of the same genetic adaptations that occur on LB agar (Fig. 3A), with lasR mutants emerging due in part to their growth advantage with amino acids. The sputum of CF patients is rich in amino acids (Ohman and Chakrabarty, 1982; Barth and Pitt, 1996), and it induces the expression of genes associated with amino acid transport and degradation.
when used as a growth medium for strain PA14 (Palmer et al., 2005). Providing additional evidence that amino acid metabolism is a target of selection, amino acid auxotrophic mutants of *P. aeruginosa* (and possibly of *Burkholderia cepacia* as well) can emerge late in chronic infection in CF (Barth and Pitt, 1995a, b). The nutritional advantage of lasR mutants may share elements with the growth advantage in stationary phase (GASP) of *Escherichia coli* mutants that overgrow LB cultures after extended incubation (Zambrano et al., 1993; Zinser and Kolter, 2004). These GASP mutants have adapted to growth in a closed system where dead cells are a food source (Farrell and Finkel, 2003), and include mutants in which decreased activity of the global regulators RpoS or Lrp confers enhanced ability to catabolize amino acids (Zinser and Kolter, 1999; 2004; Farrell and Finkel, 2003).

*Pseudomonas aeruginosa* CbrAB is a two-component regulatory system that has been suggested to balance carbon and nitrogen catabolism in part by preventing amino acid catabolic pathways from being repressed by ammonia, which can be produced at high levels as an end-product (Nishijyo et al., 2001). Such nutritional versatility may be enhanced by the increased expression of CbrB in lasR mutants that emerge during growth in amino acid-rich environments, both LB agar and the CF airway, and may contribute more to the selective advantage of lasR mutants than the enhanced ability to catabolize any single nutrient. Other aspects of the CF airway environment are likely to select for additional regulatory changes affecting *P. aeruginosa* catabolic pathways. For instance, deregulation of glucose-6-phosphate dehydrogenase (Zwf) activity is a phenotype that has been noted to emerge in sequential isolates from CF patients (Silo-Suh et al., 2006), and we detected upregulation of Zwf in isolate 5-9.6B (Table S4). Other common genetic adaptations to the CF airway (Smith et al., 2006), such as mutations in wspF that are predicted to increase levels of the bacterial second messenger cyclic di-GMP, may also reflect metabolic adaptation, given the effects on amino acid catabolism of wspF mutations in *Pseudomonas fluorescens* (Knight et al., 2006). Indeed, particularly in high-density bacterial populations, both those in the CF airway and those in laboratory models of biofilm growth, the phenotypic consequences (and adaptive significance) of mutations in global regulatory systems, such as the Las quorum-sensing system, may be nutritionally conditional (Shrout et al., 2006).

*Pseudomonas aeruginosa* lasR mutations as surrogate markers in CF

Not all *P. aeruginosa* isolates from CF patients acquire a mutation inactivating lasR, even though such mutations are common during genetic adaptation to the CF airway (Smith et al., 2006). There are many possible explanations for this observation. For instance, the lasR mutant growth advantage with amino acids could depend in part on the liberation of free amino acids by LasR-regulated proteases secreted by *P. aeruginosa* with wild-type LasR. Arguing against such an explanation, *E. coli* GASP mutants overgrow cultures by scavenging amino acids from dead bacterial cells without the need for secreted proteases. Selection pressure for loss of LasR activity could also vary in the airway depending on the presence of additional bacterial species likely to compete with *P. aeruginosa* for nutrients, such as *Staphylococcus aureus*. Indeed, there is evidence that phenylalanine, an amino acid with which *P. aeruginosa lasR* mutants have a growth advantage, can be a particularly valuable nutrient for *S. aureus* (Horsburgh et al., 2004). Perhaps the simplest explanation for why *P. aeruginosa* populations adapted to CF airways are not necessarily composed entirely of lasR mutants is that multiple adaptations exist, none of which are essential, and *P. aeruginosa* lineages with different subsets of these adaptations can arise and coexist during chronic infection.

The complexity of *P. aeruginosa* population dynamics notwithstanding, lasR mutations may serve as markers of an early stage in chronic infection of the CF airway (Hogardt et al., 2007), a stage characterized by selection pressure for mutations that enable more efficient acquisition and utilization of available nutrients. Similarly, mutations in *E. coli* that inactivate the FlhDC regulatory system (required for flagellar motility) promote colonization of the mouse intestine by enhancing catabolism of available sugars (Leatham et al., 2005). A catabolic advantage, particularly one conferring derepressed growth at high bacterial cell density (Fig. 1C), is a good candidate for an early *P. aeruginosa* adaptation to the CF airway, given that it is during early infection that bacterial population density increases (Rosenfeld et al., 2001), ultimately reaching the high densities characteristic in adult CF patients. From such a perspective, the emergence of lasR mutants during adaptation to the CF airway reflects the different demands on *P. aeruginosa* during chronic as opposed to acute infection (Nguyen and Singh, 2006): although inactivation of lasR results in loss of acute virulence determinants, it confers metabolic adaptation that may be a determinant of chronic virulence.

Just as genetic markers of cancer progression are used to direct cancer treatment (Hanahan and Weinberg, 2000), *P. aeruginosa* markers of early adaptation to the CF airway (such as lasR mutations) may be useful in predicting the course of airway disease, and predicting the responses of individual CF patients to particular antibiotic therapies. The increased tolerance to ceftazidime due to inactivation of lasR (Fig. 6D) may contribute to a selective advantage in the airway, and facilitate the subsequent acquisition of...
mutations that confer increased resistance to β-lactam antibiotics, which are commonly used to treat CF respiratory exacerbations (Giwercman et al., 1990; Salunkhe et al., 2005). Furthermore, the relative prevalence of lasR mutants in the airway is likely to influence the efficacy of new treatments targeted at inhibiting quorum sensing as a virulence determinant (Hentzer et al., 2003; Smith and Iglewski, 2003; Muh et al., 2006). Additional studies will be required to validate such predictions. The increased susceptibility of lasR mutants to fluorophenylalanine (Fig. 1B) suggests that *P. aeruginosa* adaptations to the CF airway could be used as targets for new treatment strategies, just as the use of antimetabolites in cancer therapy exploits the unregulated growth that is a hallmark of cancer cells (Hanahan and Weinberg, 2000). These treatment strategies could be tailored to the characteristics of the bacterial population in individual patients. Such directed strategies, even without eradicating *P. aeruginosa*, might slow the expansion of the bacterial population in the airway, thereby slowing the progressive decrease in lung function, and leading to increased lifespan.

**Experimental procedures**

**Bacterial strains**

The *P. aeruginosa* isolates from CF patients analysed here have been described previously (Burns et al., 2001; Smith et al., 2006): CF001, CF215 and CF716 were isolated from different infants at the ages of 3 months, 6 months and 12 months respectively (Burns et al., 2001); CF416 and CF5296 are from CF Patient 1 and have been analysed by whole genome sequencing (Smith et al., 2006); and for the remaining pairs of parentally related isolates from individual patients (3-0.8 and 3-7.3, 5-2.3 and 5-9.6B, 6-1 and 6-9.6B, 28-8.2 and 28-17.9), the designation following the hyphen represents the patient age in years (Smith et al., 2006). CF Patient 1 (Spencer et al., 2003; Smith et al., 2006) has been referred to as Patient 9 in other studies (Burns et al., 2001; Ernst et al., 2003). Strains PA14lasR::Gm (Déziel et al., 2004) and a pqsAΔpqsH double mutant (E. Déziel, unpubl. work) are derived from the laboratory strain PA14, while MP701 (Gallagher et al., 2002) is a lasR mutant derived from strain PAO1. The *P. aeruginosa* environmental isolate ENV25 was a gift from D. Speert.

**Mutant and plasmid construction**

A gentamicin-resistance cassette was inserted into lasR and into lasI in *P. aeruginosa* CF isolates by allelic exchange, using pSB219.9A and pSB219.8A, respectively, essentially as described (Beaton et al., 2002); such lasR mutants carry the designation lasR::Gm. An equivalent allelic exchange procedure (Beaton et al., 2002) was used to replace the mutated lasR gene in CF416L1 and CF1213 with a wild-type copy (as confirmed by DNA sequencing), generating CF416L1R and CF1213R respectively. This was done using a plasmid carrying wild-type lasR and flanking DNA cloned with Gateway Technology (Invitrogen) into pEXGmGW (Wolf-gang et al., 2003). Similarly, the chromosomal cbrB gene in strain CF416L1 was inactivated by allelic exchange using plasmid pEX18ApGW (Choi and Schweizer, 2005), replacing an internal fragment of cbrB (nucleotides 298–1320 in the coding sequence) with the aaC1 gentamicin-resistance gene, generating strain CF416L1cbrB::Gm. For complementation experiments, the cbrB gene and its promoter region was amplified by PCR using primers matching the upstream (5′-CGTTCTTACCAACAGGACCC-3′) and downstream (5′-TTTGAGAGGAGTGGCTGGAGA-3′) DNA. This DNA was cloned into a Gateway-compatible derivative of pMMB67E (Fortune et al., 1986), carrying either the original ampicillin-resistance gene or a gentamicin-resistance gene, and introduced into recipient cells by conjugation.

**Bacterial growth conditions**

Bacteria were grown at 37°C, either in 2–5 ml cultures with shaking incubation or on 1.5% agar (Difco Bacto Agar), unless otherwise noted. LB broth (Miller formulation, Difco) was routinely used as growth medium. Growth tests with single carbon sources used M63 salts [3 g l⁻¹ KH₂PO₄, 7 g l⁻¹ K₂HPO₄, 2 g l⁻¹ (NH₄)₂SO₄, 1 mM MgSO₄ and 0.5 mg l⁻¹ FeSO₄] supplemented with 0.1% L-phenylalanine, 0.1% L-isoleucine, 0.045% L-tyrosine or 10 mM sodium succinate (with 50 µg ml⁻¹ p-fluoro-o-phenylalanine for ampicillin susceptibilities). Growth tests with alanine as sole carbon and nitrogen source used medium composed of 0.2% L-alanine, 50 mM KH₂PO₄ (pH 7.0), 1 mM MgSO₄ and 2 µM FeSO₄. Succinate/lactamide medium is composed of 40 mM succinate, 20 mM lactamide, 50 mM KH₂PO₄ (pH 7.0), 1 mM MgCl₂ and 2 µM FeSO₄ (Wolff et al., 1991). Protease detection agar contained brain heart infusion (Difco) with 1.5% skim milk powder (Sokol et al., 1979). Cfu were determined by plating serial dilutions made in phosphate buffered saline (PBS; Gibco) onto LB agar. *P. aeruginosa* spontaneous mutants emerged as sectors in colonies growing on LB agar, cells from these sectors were purified after growth of the colonies overnight at 37°C followed by 3–16 days at 22°C. Hypermution was assayed using 300 µg ml⁻¹ rifampicin or 500 µg ml⁻¹ streptomycin essentially as described (Maciá et al., 2005), except that mutant yields were determined after incubation for 24 h. Twitching motility was assayed by stabling cells to the Petri dish bottom at the interface with the LB agar (Whitchurch et al., 2005). PMs (Bochner, 2003) were performed by BIOLOG (Hayward, CA).

**HAQ analysis**

Cells of PA14 and PA14lasR::Gm were grown on LB agar for 24 h at 37°C, followed by 48 h at 22°C. Twenty millilitres of distilled water was added to the agar surface (floating the iridescence above the lasR mutant lawn), removed after 2 min, and extracted with ethyl acetate; an additional 20 ml of distilled water was subsequently used to wash the bacterial cells off the agar surface, and this wash was also extracted with ethyl acetate. The HAQs in these two extracts were analysed by liquid chromatography/mass spectroscopy (LC/MS) as previously described (Déziel et al., 2004), with concentration determined on the basis of 20 ml total volume.
Transcriptional profiling using DNA microarrays

Bacteria from an overnight culture were used to inoculate 50 ml of LB broth to a starting A600 of 0.01 in 250 ml flasks. These cultures, shaken at 300 r.p.m., were grown to early stationary phase (A600 of 1.2). Twenty-five millilitres of the culture was centrifuged at 22°C, and total RNA was isolated using Trizol by standard procedures (Invitrogen). RNA was purified using RNase columns (Qiagen) followed by Ambion rDNase1 treatment and RNasey repurification. The absence of DNA was confirmed by PCR, and RNA integrity was validated by glyoxylate agarose gel electrophoresis (Ambion). Fluorescently labelled cDNA was prepared using 10 μg of RNA, and processed by Qiagen PCR purification.

An Agilent oligonucleotide-based (60mer) microarray was created that contained unique oligonucleotides representing all known non-redundant open reading frames among P. aeruginosa DNA sequences from the NCBI Entrez database, together with intergenic regions (at least 200 bp in length) of the PAO1 genome. cDNA hybridization was performed according to the manufacturer’s protocol (Agilent). Microarray slides were scanned using an Agilent DNA microarray scanner and Agilent Feature Extractor software by the Center for Expression Arrays at the University of Washington. Data were normalized sequentially using intensity dependent (Lowess) normalization (per spot and per chip), division by the control channel intensity (per spot), and normalization to the 50th percentile (per chip). GeneSpring software (cross gene error algorithm) was used to calculate statistically significant changes in gene expression. Microarray experiments using biological duplicate samples were performed for the comparisons between CF416 and lasR mutants were labelled with heavy ICAT reagent. The two sets of proteins were pooled, diluted in water to a final urea concentration of 1 M, and digested with sequencing-grade trypsin (Promega, Madison, WI). This tryptic peptide mixture was separated into six fractions by cationic exchange cartridges (ABI), followed by affinity purification with avidin cartridges (ABI) to enrich for ICAT-labelled peptides. After the biotin group was cleaved from the labelled product, fractions were suspended in 50 μl water with 0.1% formic acid, and analysed by microcapillary LC-MS/MS.

For quantitative protein analysis, equal amounts of soluble protein (500 μg) from each strain in the pair to be compared were labelled by heavy or light ICAT reagents according to the manufacturer’s protocol and as previously described (Han et al., 2001; Guina et al., 2003; Wu et al., 2005). Briefly, the proteins were denatured with 6 M urea and reduced with Tris(2-carboxyethyl) phosphine in labelling buffer at 22°C. Proteins from P. aeruginosa isolates with wild-type lasR were labelled with light ICAT reagent, while proteins from lasR mutants were labelled with heavy ICAT reagent. The set of proteins were cooled, diluted in water to a final urea concentration of 1 M, and digested with sequencing-grade trypsin (Promega, Madison, WI). This tryptic peptide mixture was separated into six fractions by cationic exchange cartridges (ABI), followed by affinity purification with avidin cartridges (ABI) to enrich for ICAT-labelled peptides. After the biotin group was cleaved from the labelled product, fractions were suspended in 50 μl water with 0.1% formic acid, and analysed by microcapillary LC-MS/MS.

Quantitative proteomic analysis

Bacteria from fresh single colonies on LB agar were used to inoculate 500 ml of LB broth in 1 l flasks. These cultures, shaken at 250 r.p.m., were grown to early stationary phase (A600 of 1.2). The cells were collected by centrifugation, resuspended in labelling buffer (200 mM Tris buffer, pH 8.3; 0.05% SDS; 5 mM EDTA), and broken by French Press. Unbroken cells were removed by centrifugation, and the resulting cell protein fractions were further centrifuged at 50 000 r.p.m. for 1 h at 4°C to remove the insoluble membrane proteins. The supernatants (comprising the soluble fractions) were collected and labelled with cleavable ICAT reagents (13C heavy ICAT reagent and 15N light ICAT reagent) obtained from Applied Biosystems (ABI).

Automated data analysis and matching of the peptide CID spectra to the P. aeruginosa genome database (http://www.pseudomonas.com) were performed using SEQUEST, PEPTIDEPROPHET and XPRESS software (Trans-Proteomics Pipeline version 2.8) (Han et al., 2001; Nesvizhskii et al., 2003; http://regis.systemsbiology.net/software). Spectra were filtered by standard criteria, with probability scores ≥ 0.9, XCorr ≥ 2.0, dCn ≥ 0.1, Sp = 50. Only doubly tryptic peptides were included in quantification, and the raw MS/MS spectra were manually inspected. Relative protein abundance ratios for ICAT-labelled proteins were obtained by averaging over unique peptides identified for a particular protein; the ratios were normalized by the median value of the data set. Relative protein abundance ratios greater than 2.0 were considered to reflect significant differences in protein expression.
between the compared strains. The reported data are representative data from two independent experiments, but only reproducible ratios (in the biological duplicates) are shown.

\[\text{\(\beta\)-Lactamase activity and \(\beta\)-lactam tolerance assays}\]

Plate assays for \(\beta\)-lactamase activity used ceftazidime (GlaxoSmithKline; Research Triangle Park, NC) with and without tazobactam sodium salt (Sigma-Aldrich); bacterial lawns in these assays were generated by plating 50 \(\mu\)l of a 1:10 dilution of a culture grown overnight with LB broth. To assay \(\beta\)-lactamase activity in culture supernatants, \textit{P. aeruginosa} cultures grown overnight with LB broth were passed through a filter (0.22 \(\mu\)m pore size, Millipore) and the cell-free supernatants were assayed for \(\beta\)-lactamase activity 30 min after addition of nitrocefin (Calbiochem), as described by the manufacturer, except that \(A_{480}\) was measured, and corrected by subtracting the value measured for the supernatant without nitrocefin. To determine the proportion of \(\beta\)-lactamase activity that is cell-associated, \textit{P. aeruginosa} cultures grown overnight with LB broth were diluted 1:100 in LB broth and grown for 12 h. These cultures were centrifuged and the supernatant saved. The cell pellet was resuspended in PBS and sonicated, and intact cells were removed by centrifugation. This cell lysate and the culture supernatant were each passed through a filter (0.45 \(\mu\)m pore size, Millipore), and 100 \(\mu\)l was incubated at 22\(^\circ\)C in assay buffer (1 ml total volume) with 5.16 \(\mu\)l of nitrocefin solution. Nitrocefin hydrolysis activity was measured spectrophotometrically as \(A_{480}\) (O’Callaghan \textit{et al.}, 1972), and normalized for protein concentration as measured by a modified Lowry assay (Bio-Rad).

To assay \(\beta\)-lactam tolerance, \textit{P. aeruginosa} cultures grown overnight with Mueller–Hinton broth (Difco) were diluted 1:1000 (yielding approximately 10\(^5\) cfu in 100 \(\mu\)l total volume) in wells of 96 well, round-bottom polystyrene microtiter plates (Nunc) containing serial dilutions of ceftazidime. The microtiter plates were incubated for 18 h at 37\(^\circ\)C without shaking, and MIC was determined as the lowest concentration of antibiotic for which no visible turbidity or cell pellet was observed. To reveal differences in tolerance to ceftazidime, cultures with no visible turbidity were removed from wells, and cfu was determined by plating serial dilutions.

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\section*{References}


**Supplementary material**

The following supplementary material is available for this article online:

**Table S1.** Phenotype microarray analysis of *lasR* mutant CF41L1.

**Table S2.** DNA microarray analysis of *lasR* mutants.

**Table S3.** Quantitative proteomic analysis of *lasR* mutants.

**Table S4.** Quantitative proteomic analysis of strain 5-9.6B.

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