Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of *Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa* isolates have a highly conserved core genome representing up to 90% of the total genomic sequence with additional variable accessory genes, many of which are found in genomic islands or islets. The identification of the Liverpool Epidemic Strain (LES) in a children’s cystic fibrosis (CF) unit in 1996 and its subsequent observation in several centers in the United Kingdom challenged the previous widespread assumption that CF patients acquire only unique strains of *P. aeruginosa* from the environment. To learn about the forces that shaped the development of this important epidemic strain, the genome of the earliest archived LES isolate, LESB58, was sequenced. The sequence revealed the presence of many large genomic islands, including five prophage clusters, one defective (pyocin) prophage cluster, and five non-phage islands. To determine the role of these clusters, an unbiased signature tagged mutagenesis study was performed, followed by selection in the chronic rat lung infection model. Forty-seven mutants were identified by sequencing, including mutants in several genes known to be involved in *Pseudomonas* infection. Furthermore, genes from four prophage clusters and one genomic island were identified and in direct competition studies with the parent isolate; four were demonstrated to strongly impact on competitiveness in the chronic rat lung infection model. This strongly indicates that enhanced in vivo competitiveness is a major driver for maintenance and diversifying selection of these genomic prophage genes.

[Supplemental material is available online at www.genome.org. The *P. aeruginosa* LESB58 sequence data from this study was submitted to EMBL (http://www.ebi.ac.uk/embl/) under accession no. FM209186.]

*Pseudomonas aeruginosa* is a ubiquitous organism distributed widely in the environment, including the soil and water and in association with various living host organisms. It is one of the most prevalent causes of opportunistic infections in humans and is the most common cause of eventually fatal, persistent respiratory infections in cystic fibrosis (CF) patients. It has been assumed to owe its versatility to its genetic complexity. Sequencing of four strains (Stover et al. 2000; Lee et al. 2006; Mathee et al. 2008), and molecular genetic analysis of others, has revealed an ∼6- to 7-Mb genome with ∼5500 open reading frames. Based on comparisons of the first two *P. aeruginosa* genomes sequenced, those of strains PAO1 (Stover et al. 2000) and PA14 (Lee et al. 2006) (the latter of which is the most common genotype encountered in diverse habitats in one study of 240 isolates; Wiehlmann et al. 2007), it was revealed that there is a quite highly conserved core genome representing up to 90% of the total genomic sequence; subsequent studies have revealed an extraordinary similarity of the core genome with an average nucleotide divergence of ∼0.5% (one in 200 nucleotides). Other changes that can occur include the loss of core genes through deletion or loss of expression through mutation (e.g. with the pyoverdine and O-antigen biosynthesis genes; Spencer et al. 2003).

In addition to this core genome, there are variable accessory genes, which are largely associated with genomic islands (GIs) and islets that are subject to what is termed diversifying selection, or rapid change that is presumed to be due to certain selective pressures. Some of these GIs have been well described, including a mobilizable 108-kb pathogenicity island PAPI-1 (Qiu et
that, in strain PA14, carries several regulatory genes, including \textit{pvrR} that regulates antibiotic resistance and biofilm formation, a smaller (11-kb) PA14 pathogenicity island PAPI-2 encoding the exotoxin ExoU, a 14 gene island of PAK that encodes the flagellin glycosylation machinery (Arora et al. 2001), two tandem defective phage (pyocin) islands in PA01 (but widely distributed) that are determinants of fluoroquinolone susceptibility (Brazas and Hancock 2005), and a 103-kb mobile G1 pKLC102 from clone C isolates that appears to comprise a hybrid of plasmid and phage features (Klockgether et al. 2004). While these specific instances have been studied and general features of the diversifying GIIs are well understood, there is still considerable debate as to what are the forces that shape genomic diversity among \textit{P. aeruginosa} isolates and, in particular, what selective advantages are provided by the variable accessory genes. We felt that the discovery of epidemic strains from the lungs of patients with CF provided an unprecedented opportunity to address this issue.

The widespread assumption that CF patients acquire only unique strains of \textit{P. aeruginosa} from the environment was challenged when molecular typing was used to demonstrate the spread of a beta-lactam-resistant isolate, now known as the Liverpool Epidemic Strain (LES), at a children’s CF unit in Liverpool, United Kingdom (Cheng et al. 1996). Subsequent identification of other CF epidemic strains in the United Kingdom (Scott and Pitt 2004; Lewis et al. 2005) and Australia (Armstrong et al. 2003; O’Carroll et al. 2004) indicate that transmissible \textit{P. aeruginosa} strains make a significant contribution to the infection of patients in some CF centers. LES is the most frequent clone isolated from CF patients in England and Wales (Scott and Pitt 2004) and has also been reported in Scotland (Edenborough et al. 2004). In addition, LES can cause superinfection (McCallum et al. 2001), exhibits enhanced survival on dry surfaces (Panagia et al. 2005), and is associated with greater patient morbidity than other \textit{P. aeruginosa} strains (Al Aloul et al. 2004). In two unusual cases, transmission of an LES strain occurred from a CF patient to both non-CF parents, causing significant morbidity and infections that have persisted (McCallum et al. 2002), and from a CF patient to a pet cat (Mohan et al. 2008). LES isolates, including isolate LESB58, exhibit an unusual phenotype, characterized by early (in the growth curve) overexpression of the cell-density-dependent quorum sensing regulon, including virulence-related secreted factors such as LasA, elastase, and pyocyanin (Salunkhe et al. 2005; Fothergill et al. 2007). Furthermore, LESB58 is known to be a biofilm hyperproducer (Kukavica-Ibrulj et al. 2008). Hence, LES is a successful and aggressive clone that is particularly well adapted to the CF lung. While all \textit{P. aeruginosa} isolates are intrinsically resistant to antimicrobials, like other CF isolates that cause chronic infections and are treated over time with antibiotics, LES can readily mutate to resistance to the common antibi- otics utilized in therapy (although LESB58 does not have a mutator phenotype like many other mature CF isolates, including other LES isolates). Indeed, LES was first identified because of the widespread occurrence of \textit{P. aeruginosa} isolates exhibiting cef- tazidime resistance in a clinic where cefazidime monotherapy was in routine use (Cheng et al. 1996). A survey of multiple LES isolates demonstrated that the strain can also acquire resistance to meropenem, aztreonam, tobramycin, and ciprofloxacin (Fothergill et al. 2007).

The \textit{P. aeruginosa} strains PA01 and PA14 were previously compared with LES isolate LESB58 to assess in vivo growth, infection kinetics, bacterial persistence, and localization within tissues in a rat model of chronic lung infection (Kukavica-Ibrulj et al. 2008).

The three \textit{P. aeruginosa} strains demonstrated similar growth curves in vivo but differences in lung tissue distribution and in virulence in a competitive in vivo assay. The LESB58 strain persisted in the agarose beads used to deliver bacteria into the bronchial lumen, while PA01 and PA14 strains were found to disseminate into the alveolar regions and grew as macrocolonies after 14 d post-infection.

To learn about the forces that have shaped the development of this very important epidemic strain, we sequenced here the genome of the earliest archived LES isolate, LESB58. LESB58 was obtained from a Liverpool CF patient in 1988, 8 yr prior to the first published study on the LES (Cheng et al. 1996). The sequence revealed the presence of many large GIIs, including five prophage clusters, one defective (pyocin) prophage cluster, and five nonphage islands. To determine the role of these clusters, we performed an unbiased signature tagged mutagenesis (STM) study and screening in the chronic rat lung model. The data revealed genes from the prophage clusters that strongly impacted on competitiveness in this chronic infection model, indicating that acquisition of these prophage genes contributed to the success of the LES strain.

**Results and Discussion**

**Genome sequencing**

The genome of LESB58 was fully sequenced using a combination of shotgun approaches and genome finishing techniques and is depicted in Figure 1 with statistics available in Table 1. The genome was annotated using a combination of automated methods and manual curation (see Methods), and is available through the \textit{Pseudomonas} Genome Database at www.pseudomonas.com, which represents a repository for all completed \textit{Pseudomonas} genome sequences released publicly to date.

**Virulence genes**

The LESB58 genome carries virtually all of the reported virulence genes of \textit{P. aeruginosa}. Of the 265 \textit{P. aeruginosa} virulence factor coding sequences (CDS) described for strain PA01 (Wolgast et al. 2003), all but two are present in the LESB58 genome. Clearly orthologous CDS to PA01 PA2399 (\textit{pvld}) and PA1392 were not present in PA1392, a putative nonribosomal peptide synthetase within the type I pyoverdine synthesis gene cluster. Instead, the LESB58 genome carries genes for the synthesis of a type III pyoverdine, which include a type-specific, divergent \textit{pvld} (Smith et al. 2005). Notably, there are novel duplications of pyoverdine-associated genes in the genome of strain LESB58, which carries three identical copies of the \textit{fpvA} gene (encoding the type III pyoverdine receptor) and the adjacent gene \textit{pvde} (encoding an ABC transporter). Two additional but truncated versions of \textit{pvde} are also present. PA1392 is a hypothetical protein of unknown function. Some virulence-related LESB58 CDS were divergent from strain PA01, including those matching PA1695 (\textit{pscP}) and PA2525-7 (\textit{pilABC}). The LES genome contains the type III secretion gene \textit{pseP}, but with a 10-residue deletion (\textit{5'-PTPTPTPTPTT'-3'}; position 108–117) in the predicted protein in comparison to the strain PA01 predicted protein. Further analysis of virulence was performed in the STM study described further below.

**Motility organelles**

Variations in the type IV pilin \textit{pil} locus are not uncommon (Kus et al. 2004). The LESB58 genome contained \textit{PilB} and \textit{PilC} CDS...
sharing 88% and 84% identity with PAO1 orthologs respectively, but both matched *P. aeruginosa* strain 2192 orthologs with 99% identity. The LES putative PilA was identical to a previously reported unusual PilA (GenBank accession no. AAC63060) (Pasloske et al. 1988) but shared only 32% identity with the PAO1 ortholog. Most important, however, in this regard was that the parental strain and tested clonal derivatives were completely devoid of any form of motility, including flagellin-dependent swimming motility, pilus dependent twitching motility, and viscosity-regulated swarming motility (Supplemental Table 1), consistent with the observation that unlike strains PAO1 and PA14, LES tends to remain tightly associated with the agar beads utilized in the rat chronic lung model (Kukavica-Ibrulj et al. 2008). By use of electron microscopy, we could detect neither flagella nor pili on the surface of LESB58, explaining the loss of motility. A whole *P. aeruginosa* PA14 genomic mutant library screen for deficiencies in swarming motility revealed that PA1628 mutants were less motile (E. Torfs and R.E.W. Hancock, unpubl.) and the equivalent gene in LESB58 was a pseudogene (PLES_36981/91) (for a listing of all pseudogenes identified, see Supplemental Table S2). Similarly other genes that were adjacent to the homologs of other pseudogenes (PA2023, PA2026, PA2399, PA4688, PA5454, PA5655) led to loss of swarming motility when mutated in *P. aeruginosa* PA14.

**Phenazine biosynthesis**

Phenazine compounds produced by fluorescent *Pseudomonas* species are metabolites that function in microbial competitiveness, and appear to play a role in virulence in *P. aeruginosa*. As

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**Figure 1.** Circular map of the *P. aeruginosa* LES genome. Starting from outermost circle going inwards: major (500 kb) and minor tick (100 kb) measurements of the genome with estimated location of the origin; prophage (orange) and genomic islands (green) are highlighted across all tracks; protein coding genes (blue) on plus (outer) and minus strand (inner); tRNAs (green), rRNAs (orange), and all other noncoding RNA genes (purple); signature tagged mutants (black); GC content (outer black line plot) with GC content average (gray line); and GC skew (inner black line plot) were calculated using a 10-kb non-overlapping window. Note that one region of low GC, upstream of the first noted prophage, plus additional smaller regions of low GC, contain ribosomal genes that are commonly known to have a lower GC in genomes. The location of two highly similar genomic regions of length 7.5 kb and 13.5 kb within the prophages are marked with looping purple lines, between their locations on the innermost circle. The identified prophage and GIs are distributed around the genome, but there is one notable cluster of LESGI-1, LESGI-2, and LESGI-3, reflecting the nonrandom nature of GI insertion in *P. aeruginosa* (Wiehlmann et al. 2007). Significant sequence composition bias in seven of the nine regions was computationally identified (Table 2), while GC content deviating from the average can be observed for these regions in the figure.
Identified genomic islands and prophage regions

See Methods for details.

BLAST hits and gene synteny analysis, with some validation by Ortholuge.

RNA genes 96

bSequence composition bias is indicated if the majority of the region was found to have sequence bias by either alien_hunter (Vernikos and Parkhill 2006) or the IslandPath:DIMOB (Hsiao et al. 2005) method.

The approximate start and end positions are given for those regions without PCR analysis, except for prophages 2 and 3 and LESGI-5.

with other P. aeruginosa genomes, the genome of LESB58 contained two clusters of genes encoding putative phenazine biosynthesis pathways. One cluster matched that of strain PAO1 phzA2-phzG2 (PA1899–1905) but contained phzB gene sharing greater identity to PAO1 phzB1 (PA2421). The second cluster began with orthologs to the strain PAO1 phzA1-phzB1 (PA4210-4211) but the downstream genes shared greater identity with PAO1 phzC2-phzG2.

**Table 1. P. aeruginosa LES genome statistics**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome size</td>
<td>6,601,757 base pairs</td>
</tr>
<tr>
<td>Total number of genes</td>
<td>6027</td>
</tr>
<tr>
<td>Protein coding genes</td>
<td>5931</td>
</tr>
<tr>
<td>RNA genes</td>
<td>96</td>
</tr>
<tr>
<td>Pseudogenes</td>
<td>34</td>
</tr>
<tr>
<td>Genomic islands (genes)</td>
<td>5 (214)</td>
</tr>
<tr>
<td>Prophage (genes)</td>
<td>6 (210)</td>
</tr>
<tr>
<td>PALES genes with no orthologs in:*a</td>
<td>PAO1</td>
</tr>
<tr>
<td>PA14</td>
<td>528</td>
</tr>
<tr>
<td>PA7</td>
<td>825</td>
</tr>
<tr>
<td>Any P. aeruginosa strains</td>
<td>350</td>
</tr>
</tbody>
</table>

*Orthologs were determined using a combination of reciprocal best BLAST hits and gene synteny analysis, with some validation by Ortholuge. See Methods for details.

**Table 2. Identified genomic islands and prophage regions**

<table>
<thead>
<tr>
<th>Region name</th>
<th>Integration site relative to PAO1</th>
<th>Approximate start position</th>
<th>No. of genes</th>
<th>Sequence composition biasb</th>
<th>Mobility gene(s) present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prophage 1</td>
<td>PA0611–PA0649</td>
<td>Start* 665561  End* 680385</td>
<td>19</td>
<td>No</td>
<td>None</td>
</tr>
<tr>
<td>Prophage 2</td>
<td>PA4138–PA4139</td>
<td>Start* 863875  End* 906018</td>
<td>44</td>
<td>Yes</td>
<td>Integrase</td>
</tr>
<tr>
<td>Prophage 3</td>
<td>PA3663–PA3664</td>
<td>Start* 1433756  End* 1476547</td>
<td>53</td>
<td>Yes</td>
<td>Integrase</td>
</tr>
<tr>
<td>Prophage 4</td>
<td>PA3463–PA3464</td>
<td>Start* 1684045  End* 1720850</td>
<td>48</td>
<td>No</td>
<td>Transposase</td>
</tr>
<tr>
<td>LESGI-1</td>
<td>PA2727–PA2737</td>
<td>Start* 2504700  End* 2551100</td>
<td>31</td>
<td>Yes</td>
<td>Transposase and integrases</td>
</tr>
<tr>
<td>Prophage 5</td>
<td>PA2603–PA2604</td>
<td>Start* 2690450  End* 2740350</td>
<td>65</td>
<td>Yes</td>
<td>Integrase</td>
</tr>
<tr>
<td>LESGI-2</td>
<td>PA2593–PA2594</td>
<td>Start* 2751800  End* 2783500</td>
<td>18</td>
<td>No</td>
<td>None</td>
</tr>
<tr>
<td>LESGI-3</td>
<td>PA2583–PA2584</td>
<td>Start* 2796836  End* 2907406</td>
<td>107</td>
<td>Yes</td>
<td>Integrase</td>
</tr>
<tr>
<td>LESGI-4</td>
<td>PA2217–PA2229</td>
<td>Start* 3392800  End* 3432228</td>
<td>32</td>
<td>Yes</td>
<td>None</td>
</tr>
<tr>
<td>Prophage 6</td>
<td>PA1191–PA1192</td>
<td>Start* 4545190  End* 4552788</td>
<td>12</td>
<td>Yes</td>
<td>Integrase</td>
</tr>
<tr>
<td>LESGI-5</td>
<td>PA0831–PA0832</td>
<td>Start* 4931528  End* 4960941</td>
<td>26</td>
<td>Yes</td>
<td>Integrase</td>
</tr>
</tbody>
</table>

*The approximate start and end positions are given for those regions without PCR analysis, except for prophages 2 and 3 and LESGI-5.

bSequence composition bias is indicated if the majority of the region was found to have sequence bias by either alien_hunter (Vernikos and Parkhill 2006) or the IslandPath:DIMOB (Hsiao et al. 2005) method.

**Antibiotic resistance**

The original LESB58 isolate did not demonstrate remarkable antibiotic resistance, although like other P. aeruginosa isolates that infect the lungs of individuals with CF, it is virtually impossible to eradicate once it becomes established (Hancock and Speert 2000). In such cases, initial infections are suppressed by antibiotic treatment, but over time antibiotics become increasingly less effective and resistance becomes established to one antibiotic after another. While many CF isolates acquire hyper mutator capabilities, e.g., by mutations in their mutT or mutS genes, LESB58 is not hypermutable, although subsequent isolates of this epidemic strain had acquired such status (Fothergill et al. 2007). Nevertheless the seeds for resistance development as observed in subsequent isolates are indeed present in the chromosome. The major cause of β-lactam resistance is derepression of the class-C chromosomal beta-lactamase (PA4110), and its homolog, and those of all of the accessory regulatory genes are present in the genome. Another major cause of multidrug resistance is derepression of the expression of particular efflux pumps, of which P. aeruginosa has a wide variety. Mutations in certain efflux pump genes were observed. For example, the positive regulator of MexEF-OprN, mexT (PA2492 homolog), was a pseudogene in LESB58, while the mexF (PA2494) gene is present but mutated, leading us to suspect that the MexEF-OprN efflux system was minimally operative and perhaps not derepressible in the LES. Similarly, MexZ (PA2020) was also a pseudogene. However, the major efflux pump contributing to intrinsic and mutational resistance, MexAB-OprM, and the ancillary system, MexCDO-OprJ, were intact. In other LES isolates exhibiting greater antimicrobial resistances, depression of AmpC and mutations in mexT and mexZ, implicated in up-regulation of the MexAB-OprM and MexXY efflux pumps, respectively, have been identified (Salunkhe et al. 2005). Of the 31 PAO1 CDS annotated as functional class “antibiotic resistance and susceptibility” in the Pseudomonas Genome Database, only PA2818 (arr), a putative aminoglycoside response regulator, was absent from the genome of the LES.

**LES bacteriophage gene clusters**

Isolate LESB58 contained six prophage gene clusters, termed here as prophages 1–6 (Table 2; Fig. 2; Supplemental Table 3), of which...
was similar to a region of the phages, LES prophage 3 shared 82.2% identity with a region of prophage 2 with homology to known phages, LES prophages 4 and 5 carried putative cl genes that were quite divergent from those of the related phages D3 and D3112, respectively (Supplemental Fig. 1).

The LES prophage 6 gene cluster was similar to the genome of bacteriophage Pf1 (Hill et al. 1991). It has been suggested that Pf1 genes might be important in CF infections, in that Pf1 genes are up-regulated under conditions of reduced oxygen supply (Platt et al. 2008), implicated in the augmentation of the antimicrobial efficacy of antibiotics (Hagens et al. 2006), and play an active role in the activity and adaptation of P. aeruginosa populations biofilms (Webb et al. 2003, 2004; Sauer et al. 2004; Mooij et al. 2007). However, since most clinical isolates carry Pf1-like phages, these activities are not restricted to successful CF strains such as the LES (Finnan et al. 2004).

Ability of four prophage gene clusters to produce functional phage particles

To determine the activity of the LES prophages 2–5 gene clusters, we designed PCR assays to detect phage DNA in culture supernatants after induction with subinhibitory concentrations of norfloxacin. These PCR assays would only be positive if there were phage particles present because they require a circularized form of the genome to be present for observation of a product. Similarly, we performed PCR assays to determine the presence of these sequences in boiled cells. The distribution of phage sequences for LES prophages 2–5 was determined in 26 LES clinical isolates (Table 3). Prophages 3 and 4 were found in all 26 tested isolates, while prophage 2 was identified in 23 of the tested 26 isolates. Although for each of these isolates phage particle production was observed in some clinical isolates, there was substantial variation with 12, 18, and 25 of the isolates tested producing prophage 3, 2, and 4 particles, respectively. In contrast, the D3-like prophage 5 gene cluster was only carried by a minority (eight of 26) of LES isolates, although each of these were able to produce phage particles. We were able to isolate LES prophages 2 and 3 by plaque assay on P. aeruginosa strain PAO1 lawns and have observed bacteriophage particles by electron microscopic analysis of both plaque suspensions and culture supernatants (J.L. Fothergill and C. Winstanley, unpubl.)

LES GIs

Many GIs have been identified in P. aeruginosa strains in previous studies, including PAGI-1 (Liang et al. 2001), PAGI-2 and PAGI-3 (Larbig et al. 2002), PAGI-4 (Klockgether et al. 2004), PAPI-1 and PAPI-2 (He et al. 2004), and pKLC102 (Klockgether et al. 2004). Several other unpublished GI sequences have been deposited

Table 3. Distribution of phage gene sequences among LES isolates

<table>
<thead>
<tr>
<th>Target</th>
<th>Presence by PCR product as a function of the number of tested isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells (genomic)</td>
</tr>
<tr>
<td>LES prophage 2</td>
<td>23/26</td>
</tr>
<tr>
<td>LES prophage 3</td>
<td>26/26</td>
</tr>
<tr>
<td>LES prophage 4</td>
<td>26/26</td>
</tr>
<tr>
<td>LES prophage 5</td>
<td>8/26</td>
</tr>
<tr>
<td>Control PCRs</td>
<td>—</td>
</tr>
</tbody>
</table>

*Also tested for PCR products by screening for circularized phage genomes with the same strains showing positive results.

Figure 2. Phage clusters identified in LESB58 with significant similarities and positioning of STM mutants after in vivo screening.

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into GenBank that extend the PAGI nomenclature from PAGI-5 (EF611301) to PAGI-11 (EF611307). The observed LESB58 large GIs are summarized in Table 2 and described in greater detail in Supplemental Table 3. Previous studies using subtractive hybridization identified representative sequences from these islands and reported on their prevalence among LES and non-LES CF isolates (Smart et al. 2006b). Basically, these GIs were generally common to all LES isolates (with the lowest frequency observed being 86% for LESGI-5, the only island unique to LES isolates).

Only two of the five GIs identified within the LES strain showed similarity to any previously identified P. aeruginosa island, with the last 67 kb of the 110-kb LESGI-3 island showing similarity to PAGI-2, PAGI-3, PAGI-5, and PAPI-1 (Fig. 3), while LESGI-4 shared 46% identity with PAGI-1 over its entire length. This is consistent with previous evidence that GIs are a major source of novel genes for a genome (Hsiao et al. 2005; Tettelin et al. 2005). As previously noted, pKLC102 and the related PAPI-1 were not novel genes for a genome (Hsiao et al. 2005; Tettelin et al. 2005). LESGI-1 was inserted at a tRNA locus, contained phage- and transposon-related CDS and included the LESF9 PCR diagnostic marker (Smart et al. 2006b; Fothergill et al. 2008). However, it also contained several CDS sharing similarity with predicted proteins from nonpsychrohemobacter such as the thermophilic anaerobe Clostridium thermocellum and the marine bacteria Marinobacter sp. Although mostly matching hypothetical proteins of no known function, the island included homologs of regulatory proteins, restriction-modification proteins, an ATPase, and a sensor kinase. This island included PALES23591, which contains the LESF9 marker, although it is not unique to LES isolates (Smart et al. 2006b).

LESGI-2 contained a pyoluteorin biosynthesis gene cluster (pltMRLABCDEFGZHIJKNO) sharing 99% nucleotide sequence identity with a cluster from Pseudomonas sp. M18 (AY394844) but containing a frameshift mutation in pltB. Pyoluteorin has anti-fungal activities (Bender et al. 1999) and may play an important role in the ability of plant associated pseudomonads, such as P. fluorescens, to suppress a variety of plant diseases (Nowak-Thompson et al. 1999). Interestingly, in LESB58, as previously found in the genome of Pseudomonas sp. M18, the island was adjacent to a PA2593-like CDS. LESGI-3 was related to the PAGI-2 GI of Clone C (Larbig et al. 2002; Klockgether et al. 2004) with an alternative cargo region containing multiple putative transport proteins. It includes the PCR diagnostic marker PS21 (Parsons et al. 2002) that has homology with a putative mercuric reductase gene (PLES26321), although the island has been identified in some non-LES strains (Lewis et al. 2005). LESGI-4 was related to the GI PAGI-1 (Liang et al. 2001).

LES GI-5 was a novel island containing genes that largely match those of organisms other than P. aeruginosa and including a putative phage integrase and plasmid replication genes (Supplemental Table S1). Aside from those associated with mobile elements, most predicted protein BLASTP matches shared <50% identity. The exceptions were two hypothetical proteins, one from Dechloromonas aromatica that is associated with aquatic and sediment habitats and one from P. syringae pv. tomato.

**Signature-tagged mutagenesis of LESB58**

Signature-tagged mutagenesis (STM) is a well-defined method for determining, in a relatively unbiased manner, the importance of specific genes in vivo growth, through the relative ability of mutants to survive in animal models of infection. Since LES is an extremely robust epidemic isolate in CF and since it previously demonstrated a competitive advantage over other P. aeruginosa strains in relevant animal models of infection (Kukavica-Ibrulj et al. 2008), we investigated here the basis for its success by STM. The PCR-based STM of P. aeruginosa strain LESB58 involved both the construction of a library of signature tagged mutants in vitro and an in vivo selection step as outlined in Supplemental Figures S2 and S3. The in vitro construction of plate libraries of mutants containing specific, defined DNA tags for PCR screening. P. aeruginosa LESB58 mutants led to 96-deep-well microtitre plates, each containing 96 STM mutants (total of 9216 STM mutants). Analysis by Southern blot of 50 randomly selected LESB58 STM exconjugant strains showed unique insertions; sequencing of the junction DNA neighboring the inserted tet gene confirmed random, unique insertions in genes scattered around the LESB58 chromosome. Subsequently, in vivo screening was performed to identify P. aeruginosa strain LESB58 genes implicated in lung infection, using pools of 48 mutants per animal and the rat agar bead model of chronic lung infection, which is considered to be the most relevant model of CF lung infections. Output pools were analyzed for the retention or loss of each mutant by PCR 7 d post-infection. To confirm the identity of each STM mutant, 24 signature tag PCR amplifications were done to give DNA products of a specific length of 820 bp and 980 bp for the TnsTc and TnsTc GFP, visible in agarose gels. Each STM mutant selected as defective for growth in vivo was reconfirmed by PCR. LESB58 STM mutants having a visible positive PCR product in vitro and no PCR product after in vivo passage were retained for further analysis (e.g., Table 4).

**Figure 3.** Genomic islands identified in LESB58 with significant similarities and positioning of STM mutants after in vivo screening.
Of the >60 LESB58 STM mutants that were attenuated in lung infection, 47 provided an unambiguous sequence location (Table 4) by mapping of the insertion points of the transposon. Six of these genes were also found in a previous STM screening using strain PAO1 (Table 4). DNA sequencing revealed insertions in most known functional gene classes. These included insertions in genes encoding products or processes previously implicated in pathogenesis of *P. aeruginosa*, such as the type III secretion protein Psch, a haem iron uptake receptor PhuR, TolA, the fimbrial usher CupA3, the alginate biosynthesis protein MucD, and two transcriptional regulators PA2583 and PA2020. Insertions in genes involved in the biosynthesis of type III pyoverdine (*pvdE*) and pyochelin (*PA4226*) were identified, emphasizing the importance of both siderophores.

In vivo analysis of STM mutants having insertions in prophage gene clusters

To assist in understanding the basis for the successful colonization of the LES in CF patients, the level of attenuation in vivo was determined for three STM mutants having insertions in LES prophages 2, 3, and 5 and one STM mutant in the unique LES GI, determined for three STM mutants having insertions in LES prophages 2, 3, and 5 and one STM mutant in the unique LES GI.
In vitro growth was assessed for each of these STM mutants in mixed cultures with the wild type (in vitro competitive index [CI]) to confirm that these mutants did not affect in vitro growth and were not outcompeted in vitro by the wild-type LESB58 strain, yielding an in vitro CI of ∼1.0 after 18 h in BHI broth. This contrasted with the results when competition was assessed in vivo, for which the mutants were mixed with the wild-type strain LESB58 and grown in the rat lung infection model for 7 d. As depicted in Figure 4, mutants with insertions in both prophages 2 and 5 caused a severe defect in growth and maintenance in vivo, which gave a significant 16- to 58-fold decrease of CFUs in rat lungs with CI values of 0.061 and 0.017, respectively. Mutants in prophage 3 and LESGI-5 could be partially maintained in lung tissues with approximately sevenfold decreases in growth in vivo.

Basis for the colonial nature of the LESs in CF patients.

The genomes of P. aeruginosa strains PAO1 (Stover et al. 2000) and PA14 (He et al. 2004) and two other CF isolates (Mathee et al. 2008) have been completed and annotated. In addition to the LES presented here, several other P. aeruginosa strains are currently being sequenced (pacs416 [early isolate], pacs416 [late isolate], pacs5296, PA7, PACS2, and PK6) (http://www.genomesonline.org). The genome of P. aeruginosa exhibits a mosaic structure (Ernst et al. 2003) and is composed of a “core genome” (∼90%) and an “accessory genome” (∼10%). The latter includes gene clusters involved in determining O-serotype (Raymond et al. 2002), flagellin type (Arora et al. 2001), type IV pili (Kus et al. 2004), siderophore production (Spencer et al. 2003), as well as genomic/pathogenicity islands (Liang et al. 2001; Larbí et al. 2002; He et al. 2004; Klockgether et al. 2004; Gal-Mor and Finlay 2006) and prophages. Although many of the known virulence genes are carried within the core genome of P. aeruginosa (Wolfgang et al. 2003), genes from the accessory genome can contribute to pathogenicity. The genome of LESB58, like those sequenced previously, carries the core genome, including the vast majority of recognized virulence genes of P. aeruginosa. The genomic variations lie largely within five prophages and one defective prophage and five large GIs, a few of which are related to those found in other strains of P. aeruginosa.

Extensive genome plasticity has been reported for P. aeruginosa clinical isolates, with phage sequences making a significant contribution to horizontal gene transfer, leading to sequence diversity (Shen et al. 2006). Indeed, it has been suggested that integrase-driven instability plays an important role in bacterial genomic evolution (Manson and Gilmore 2006). Furthermore, it has been demonstrated that bacteriophages can drive diversification of P. aeruginosa (Broekhart et al. 2005). More than 60 temperate phages have been isolated from P. aeruginosa (Akhtar et al. 1984; Wang et al. 2004), and many have been genome sequenced.

It is well known that P. aeruginosa pathogenesis involves a variety of well-known core genome functions (e.g., Type II and III secretion, iron transport, etc.), as well as other functionally important “accessory” gene clusters determining O-serotype, flagellin type, type IV pili, and siderophore production (although these are only named accessory genes because of their sequence divergence and it is arguable that these are really core functions). Here we have shown for one of the few well-characterized “epidemic” strains of P. aeruginosa that the success of this organism, permitting it to be retained in an infection model relevant to CF, requires genetic information encoded on three prophages and one GI. This then sheds some light on the crucial nature of the flow of genetic information through the accessory genome in such critical functions as the ability of an organism to grow successfully in a host possessing multiple mechanisms for impeding bacterial survival.

In many ways, the LES probably owes its success as a pathogen to its ability to colonize the lungs of CF patients. It would not be at all advantageous to the organism to initially adopt too aggressive a stance in dealing with its host since this would inevitably result in either rejection of the pathogen or killing of the host, essentially biting the hand that feeds it. Thus the LES strain has lost its flagella, and flagellin is the major Toll receptor (TLR) agonist that Pseudomonas uses to induce an inflammatory response in epithelial cells (Bloomke et al. 2008); this also explains the tendency of the LES to remain localized to the agar beads used in the rat lung chronic infection model used here, unlike strains PAO1 and PA14 that tend to disseminate. This unaggressive behavior may in turn explain how this strain might avoid over-excitation of a reactive inflammatory response and the consequent ability of the LES strain to outcompete both the PAO1 and PA14 strains (Kukavica-Ibrulj et al. 2008). Other features that would appear to favor colonization of the lung would be the proclivity of the LES to form biofilms, and the role of MucB in successful colonization in the rat lung model (Table 4) would also favor this conclusion. Successful colonization without overt induction of inflammation would then provide a platform from which other counter-measures could be launched, including the induction of the mutator state that permits more rapid adaptation to antibiotic resistance and possibly other favorable adaptations also. Its initial success in colonization of the lung and its ability to induce quorum regulation at lower cell densities permit it to induce a number of enzymes that will remodel its local environment, and this is responsible for the overall enhanced aggressiveness of this strain. Thus the strategic approach of this organism could be described as “passive aggressive.”
It has been demonstrated that *P. aeruginosa* virulence is combinatorial (Lee et al. 2006). The studies described here indicate an ability to successfully establish colonization in what is usually a protected niche, the lung, indicating that this too involves a combinatorial process and involves both the core genome and key prophage and GI genes from the “accessory” genome to increase competitiveness.

**Methods**

**Bacterial strains, plasmids, media, and growth conditions**

*P. aeruginosa* LESB58 represents the earliest archived isolate of the LES epidemic strain as described above. The pUTmini-Tn5 Tc and pUTmini-Tn5 TcGFP were used for insertion mutagenesis (de Lorenzo et al. 1990) and pTZ18R for cloning of chromosomal DNA (GE Healthcare). Unless otherwise indicated, *Escherichia coli* and derivatives were grown in Tryptic Soy Broth (TSB), Brain Heart Infusion (BHI) broth for *P. aeruginosa* strain LESB58, or Mueller Hinton agar (MHA) (Difco). When needed, these media were supplemented with 1.5% of Bacto agar (Difco), ampicillin (Ap, 100 µg/mL for *E. coli*), piperacillin (Pip, 100 µg/mL for *P. aeruginosa* LESB58), or tetracycline (Tc; 20 µg/mL for *E. coli* or 25 µg/mL for *P. aeruginosa* LESB58; Sigma-Aldrich). Restriction enzymes, T4-DNA ligase, T4-DNA polymerase, and T4 polynucleotide kinase were purchased from New England Biolabs (Mississauga) and used in standard procedures (Sambrook and Russell 2001). HotStart TAQ DNA polymerase was from Qiagen.

**Preparation of genomic DNA**

DNA was extracted from strain LESB58 using a simple lysis procedure followed by caesium chloride-ethidium bromide gradient ultracentrifugation (Winstanley and Hart 2000). Following removal of the chromosomal DNA band using a syringe, ethidium bromide was extracted using isopropanol, and the DNA was dialyzed against 10 mM Tris-HCl (pH 8.0) to remove the caesium chloride.

**DNA sequencing**

DNA was fragmented by sonication, and several libraries were generated. The whole genome was sequenced to a depth of 9 × coverage from pOTT12 (insert size 2–3 kb) and pMAQSac (insert size 3–12 kb) small insert libraries using dye terminator chemistry on ABI3730 capillary sequencers. End sequences from larger insert plasmid (pBACehr, 5–18 kb insert size) libraries were used as a scaffold. The sequence was assembled and finished as described previously (Young et al. 2006). The *P. aeruginosa* LESB58 genome sequence was submitted to EMBL under the accession number FM209186.

**Genome annotation**

Coding sequences (CDS) were predicted using Glimmer3 (Delcher et al. 2007) and were assigned LES locus identifiers consisting of a “PLES,” prefix followed by five digits that are incremented in multiples of 10 to allow for additional CDS or non-coding RNAs. Orthologs in PA14 and PAO1 were identified for each LES CDS or noncoding RNA using a reciprocal best BLAST approach coupled with synteny and Orthologue analysis: In particular, each LES CDS was used as the query input for a FASTA search with either PA14 or PAO1, using an identity cutoff of 30% that covered at least 80% of the query and hit. The relaxed 30% cutoff was used to capture possible cases of substantial gene divergence, and the following methods were used to eliminate cases of nonorthologous homologs. If the original LES CDS was identified as the top hit using the same search as for the PA14 or PAO1 top hit, then the top hits were considered probable orthologs. In cases where multiple top hits with the same score were identified, gene synteny, from whole genome alignments obtained with the program Mauve (Darling et al. 2004), was used to identify the most probable ortholog. Orthologs were additionally characterized using Orthologue (Fulton et al. 2006). LES genes with identified orthologs in either PAO1 or PA14, with the most recent annotations from www.pseudomonas.com (Winsor et al. 2005), were transferred automatically. Gene annotations from PAO1 were selected for transfer over PA14 in cases where LES genes had orthologs in both, due to the higher level of updated manual curation of the PAO1 genome. LES CDS that did not have an identified ortholog in PA14 or PAO1 were manually annotated based on significant BLAST matches from the NCBI nr database. Protein subcellular localization and COGs were predicted for each LES CDS using PSORTb 2.0 (Gardy et al. 2005) and RPS-BLAST (Marchler-Bauer et al. 2002), respectively.

**GI identification**

GIs and prophage regions were identified or, for those previously annotated, further refined by searching for clusters of genes (more than eight CDS) in the LES strain that had no homologs in other *P. aeruginosa* completely sequenced genomes, using Island-Pick (Langille et al. 2008). Abnormal sequence composition was calculated using alien_hunter/IVOM (Vernikos and Parkhill 2006), IslandPath/DINUC, and IslandPath/DIMOB (Hsiao et al. 2005) to provide supporting evidence, since alien_hunter is the island predictor with highest recall, while the other methods have higher overall accuracy (Langille et al. 2008). Also, the presence of genes associated with DNA mobilization (transposases, integrases, etc.) was determined by manual inspection of the gene annotations.

**PCR assays for detection of bacteriophages**

To induce phage lytic activity from lysogens, LES isolates were grown in the presence of subinhibitory concentrations of norfloxacin (determined based on minimal inhibitory concentration). The culture supernatants were filtered (0.2 µm) to remove bacteria, and DNase I treated to remove contaminating DNA. PCR assays were then used to identify each of the prophage genes in both boiled suspensions of bacteria (to identify prophages within the genome) and boiled culture supernatants (to identify DNA in phage particles). For LES, prophage 2 and prophage 3 primers were designed reading out from terminal regions so that only circularized DNA can yield amplicons. The primers and PCR amplification conditions used can be obtained from the first author on request.

**Construction of STM mutant libraries of strain LESB58**

The nucleotide sequence of the 24 oligonucleotides used for construction of signature tags and sequence of the universal primers for PCR-based STM have been described previously (Sanschagrin et al. 2008). The two sets of plasmids constructed were transformed into *E. coli* S17-1(ppir) (Simon et al. 1983) by electroporation and transformants selected on MHA supplemented with Ap (100 µg/mL) and Tc (20 µg/mL). Single colonies were purified and screened by PCR. Bacterial cells were boiled for 5 min and lysates transferred to tubes containing PCR buffer, 200 µM dNTPs, and 10 pmol of each primer with 2.5 U HotStart TAQ DNA polymerase. Touchdown PCR from 65°C to 55°C was performed using specific 21-mers in combination with the pUT-TcR-ispecific primer from the tet gene. *E. coli* S17-1 ppir containing pUTmini-Tn5Tc tagged plasmids was used as a donor for conjugation.
gal transfer into the recipient \textit{P. aeruginosa} strain LESB58. Exconjugants were selected on BHI and \textit{Tc}. Colonies resistant to \textit{Tc} (reflecting the presence of mini-Tn5 in the chromosome of \textit{P. aeruginosa}) were tooth-picked onto BHI agar plates with Pip (to exclude bacteria having the cointegrated suicide donor plasmid pGP704). Colonies of exconjugants were grown in 96-well microtiter plates with \textit{Tc}; one mutant from each library was picked to form pools of 96 unique tagged mutants (forming the in vitro input pools).

### Screening of \textit{P. aeruginosa} STM mutants in the rat lung

The previously described chronic rat lung model of infection (Cash et al. 1979) was used and optimized for preparation of beads and for screening pools of 96 mutants in vivo. Bacterial cultures for wild-type LESB58 and each STM mutant strain (\textit{Tc}, 45 \mu g/mL) were grown into 2 mL of BHI in deep 96-well plates overnight at 37°C to an optical density at 600 nm of 1, equivalent to 2 x 10^9 colony forming units (CFU)/mL, and pooled. A 0.5 mL aliquot of each pooled culture was washed twice by centrifugation at 7200 rpm for 2 min with the same volume of phosphate buffered saline (PBS) (1.37 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.3 mM KH_2PO_4 at pH 7.4). All other procedures were as described previously (Sanschagrin et al. 2008). Agar beads containing the pooled bacteria were conserved at 4°C and, based on previous experience, were available for use for up to 1 mo.

### Determination of CFU in agar beads and in vivo screening

To determine the bacterial content of agar beads, a 1 mL aliquot was added to 9 mL of PBS and homogenized with a Polytron (Kinematica AG) for 30 sec at maximum speed. One hundred microliters was serially diluted to 10^-4 and plated on BHI agar followed by overnight incubation at 37°C for up to 48 h to determine CFU.

Animals were used according to the protocol approved by the Laval University ethics committee for animal treatment. A group of male Sprague-Dawley rats (500 g; Charles River, Canada) were utilized for screening and each was injected with a pool of 48 mutants from the \textit{P. aeruginosa} LESB58 STM libraries. The animals were anaesthetized using isofluorane and were inoculated by intubation with a total dose of 7.2 x 10^8 CFUs of the pooled mutants in agar beads, injected into rat lungs using a 1-mL tuberculin syringe containing 120 mL of bead slurry. After 7 d post-infection, animals were sacrificed and lungs collected and homogenized with a Polytron and total CFUs determined by plating serial dilutions on BHI agar and on selective media (BHI, \textit{Tc} 25 \mu g/mL) after 48 h at 37°C to an optical density at 600 nm of 1, equivalent to 2 x 10^6 colony forming units (CFU)/mL, and pooled. A 0.5 mL aliquot of each pooled culture was washed twice by centrifugation at 7200 rpm for 2 min with the same volume of phosphate buffered saline (PBS) (1.37 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.3 mM KH_2PO_4 at pH 7.4). All other procedures were as described previously (Sanschagrin et al. 2008). Agar beads containing the pooled bacteria were conserved at 4°C and, based on previous experience, were available for use for up to 1 mo.

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### Selection of STM mutants by comparative PCR

An aliquot of each input pool was boiled for 10 min, spun down, and 10 \mu L of supernatant recovered for PCR analysis. After in vivo passage, lung tissues were recovered by dissection and homogenized with a Polytron homogenizer in 10 mL of sterile 1 x PBS in a 50-mL Falcon tube. One hundred microliters of homogenized tissues was plated on BHI agar plates containing 25 \mu g/mL \textit{Tc}; after growth, 10^4 colonies recovered from a single plate were resuspended in 1 mL of PCR buffer (the in vivo output pool). The in vivo output pool was subsequently boiled for 10 min, the remaining cells removed by centrifugation, and 10 \mu L of supernatant used in PCR analysis as described previously. LESB58 STM mutants that were present in the in vitro input pool and absent from the in vivo output pool were retained for further analysis by independent PCR with specific primers.

### Cloning and analysis of disrupted genes in LESB58 STM mutants

The insertion point of the mini-Tn5 in mutants of interest was identified by cloning the transposon resistance marker \textit{Tc} and by sequencing the flanking LESB58 chromosomal DNA. Chromosomal DNA (5 \mu g) from each \textit{P. aeruginosa} LESB58 attenuated mutant was isolated using the Qiagen genomic DNA extraction kit and sequenced as described previously (Sanschagrin et al. 2008). DNA sequences obtained were assembled and subjected to database searches using a Silicon Graphics Origin2000 computer via BLAST in the GCG Wisconsin package, along with GeneFinder and GeneMark. Similarity searches with the \textit{P. aeruginosa} genome were done using the \textit{P. aeruginosa} PA01, PA14, and LESB58 databases (http://www.pseudomonas.com). STMs were mapped to the LES genome using BLASTN with an E-value cut-off of 0.001. Short spurious matches were manually removed, while significant matches were mapped to overlapping genes.

### Competitive index analysis

For in vivo CI assays, bacteria were enmeshed into agar beads and the rat model of chronic lung infection was used as described above (Cash et al. 1979; Sanschagrin et al. 2008). Total CFU for mutants and the wild-type LESB58 strain CFU was determined on BHI plates, after 48 h at 37°C, while the LESB58 STM mutants CFU were differentiated on BHI with \textit{Tc}, and these values used to calculate the input ratio of LESB58 STM mutant to the wild-type LESB58 strain. Output ratios were calculated by differential counts on BHI agar and selective media (BHI, \textit{Tc} 25 \mu g/mL) after in vivo passage of the mixture of wild type with each STM mutant in the same rat lung. The CI was defined as the CFU output ratio of mutant when compared to wild-type strain, divided by the CFU input ratio of mutant to wild-type strain. The geometric mean CI was calculated for animals in the same group with experiments being done in at least three animals. Each in vivo competition experiment was examined for statistical significance by the Student’s two-tailed t-test.

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