In Vivo Growth of *Pseudomonas aeruginosa* Strains PAO1 and PA14 and the Hypervirulent Strain LESB58 in a Rat Model of Chronic Lung Infection

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*Pseudomonas aeruginosa* chronic lung infections are the major cause of morbidity and mortality in cystic fibrosis (CF) patients. The *P. aeruginosa* strains PAO1 and PA14 were compared with the Liverpool epidemic strain LEB58 to assess in vivo growth, infection kinetics, and bacterial persistence and localization within tissues in a rat model of chronic lung infection. The three *P. aeruginosa* strains demonstrated similar growth curves in vivo but differences in tissue distribution. The LEB58 strain persisted in the bronchial lumen, while the PAO1 and PA14 strains were found localized in the alveolar regions and grew as macrocolonies after day 7 postinfection. Bacterial strains were compared for swimming and twitching motility and for the production of biofilm. The *P. aeruginosa* LEB58 strain produced more biofilm than PAO1 and PA14. Competitive index (CI) analysis of PAO1, PA14, and LEB58 in vivo indicated CI values of 0.002, 0.0002, and 0.14 between PAO1-PA14, PAO1-LEB58, and LEB58-PA14, respectively. CI analysis comparing the in vivo growth of the PAO1 ΔPA5441 mutant and four PA14 surface attachment-defective (sad) mutants gave CI values 10 to 1,000 times lower in competitions with their respective wild-type strains PAO1 and PA14. *P. aeruginosa* strains studied in the rat model of chronic lung infection demonstrated similar in vivo growth but differences in virulence as shown with a competitive in vivo assay. These differences were further confirmed with biofilm and motility in vitro assays, where strain LEB58 produced more biofilm but had less capacity for motility than PAO1 and PA14.

*Pseudomonas aeruginosa* is a versatile and ubiquitous opportunistic pathogen infecting humans, animals, insects, and plants. It is considered a leading cause of nosocomial infections in hospital-acquired pneumonia, in immunocompromised individuals, and in individuals with cystic fibrosis (CF). It produces a variety of both cell-associated and extracellular virulence factors coordinately regulated by density-dependent cell-cell communication known as quorum sensing (15, 20). In addition, its motility by swimming, swarming, and twitching and its capacity of forming a biofilm are considered a leading cause of nosocomial infections in hospital-acquired pneumonia, in immunocompromised individuals, and in individuals with cystic fibrosis (CF). It produces a variety of both cell-associated and extracellular virulence factors coordinately regulated by density-dependent cell-cell communication known as quorum sensing (15, 20).

Most laboratories have been using a limited number of *P. aeruginosa* prototype strains for various studies. The *P. aeruginosa* PAO1 strain is a prototype used in many laboratories for many years, and PA14 was a human isolate that is now used as a reference strain because it has a wide host spectrum for studies of virulence. LEB58 is a hypervirulent human CF isolate. The specific features for each of these three strains are summarized in Table 1. The PAO1 strain is the standard laboratory and genetic reference strain, with a completely sequenced 6.3-Mb genome containing 5,570 annotated open reading frame (ORFs) (52). A highly virulent clinical isolate, UCBPP-PA14 (PA14) was identified as a “multihost” pathogen capable of infecting animals (in a burned mouse model), plants, insects, and nematodes (34, 44). Genetic and genomic analysis of the PA14 genome (6.5 Mb) identified pathogenicity islands and an extensive degree of conservation of virulence genes, suggesting a capability of infecting various hosts. Two pathogenicity islands of 108 and 11 kb, called PAPI-1 and PAPI-2, respectively, were identified as being unique to PA14 and absent in the PAO1 genome (23). Most of the genes within these islands are homologous to known genes found in other human and plant bacterial pathogens. For example, PAPI-1 carries a complete gene cluster predicted to encode a type IV group B pilus, a well-known adhesin absent in PAO1. In PA14, 19 PAPI-1 ORFs were found to be necessary for virulence in plants or in animals; 11 ORFs are required for both (Table 1).

The large set of “extra” virulence factors encoded by both pathogenicity islands may contribute to the increased promiscuity of the highly virulent PA14 strain. The genome of PA14 has been sequenced, and a draft version of the PA14 annotation is available at http://pga.mgh.harvard.edu/Parabiosis and has been deposited in GenBank (accession no. CP000438) (33).
A highly virulent epidemic strain (LES) was first identified in the Liverpool CF clinic center (9) and was further recognized for its epidemic nature by transfer between CF patients and from CF patients to non-CF relatives, causing significant morbidity (35). Furthermore, there is greater morbidity among CF patients colonized with the LES clone than among those carrying nonepidemic strains of P. aeruginosa (1). Compared with the PAO1 strain, the highly transmissible and aggressive LES strain displays enhanced virulence, a wider spectrum of antibiotic resistance, and presumably a better adaptation to the CF lung (46). The success of LES isolates in lung colonization may be due to the prior acquisition of genes or pathogenicity islands (40), to transcriptional variations in the level of gene expression, or to a combination of both. Such changes contribute to greater colonization and/or transmissibility of the LES strains, enhancing their ability to cause chronic infections in CF patients, and to enhanced virulence, manifesting itself in infections of non-CF parents.

The genome of P. aeruginosa displays a mosaic structure, with all strains possessing a highly conserved backbone referred to as the core genome, including recognized virulence factors (46). Variations between strains include the presence or absence of genomic islands, which can partially explain differences in virulence.

In this study, we examined the capacities of three different P. aeruginosa strains to initiate and maintain a chronic infection in the rat lung model by following in vivo growth up to 14 days. Bacteria were localized and their distribution in lung tissues determined using histological and immunofluorescence methods. We also examined bacterial motility and the capacity for the production of biofilm. The competitive indexes (CIs) between wild-type strains and several mutant strains, including PAO1ΔPA5441 and four PA14 surface attachment-defective (sad) mutants, were also determined. Previously, the PAO1Δ PA5441 mutant was identified as being attenuated in vivo by signature-tagged mutagenesis screening (42), and PA14 sad mutants have been shown to produce reduced biofilm levels (38). We used these mutants as negative controls in order to validate in vivo CI analysis and for measurement of the expression of virulence factors in vitro.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 2. Unless otherwise indicated, P. aeruginosa and Escherichia coli strains were grown in tryptic soy broth or Mueller-Hinton broth (Difco, BD, Sparks, MD). When needed, these media were supplemented with 1.5% Bacto agar and the following antibiotics at the indicated concentrations: gentamicin (Gm), ampicillin (Ap), kanamycin (Km), tetracycline (Tc) (Sigma-Aldrich, Oakville, Ontario, Canada), or carbenicillin (Cb) (Invitrogen, Life Technologies, Burlington, Ontario, Canada). Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and T4 polynucleotide kinase were purchased from New England Biolabs (Mississauga, Ontario, Canada) and used in standard procedures (47). HotStart Taq DNA polymerase was from Qiagen (Mississauga, Ontario, Canada), and PCRs were performed in an iCycler thermocycler (Bio-Rad, Mississauga, Ontario, Canada).

**Preparation of bacteria for in vivo experiments.** (i) Preparation of agarose-embedded bacteria for determination of individual kinetics and CIs. Preparation of the individual P. aeruginosa strains or wild-type–mutant mixtures in agarose beads was modified from a previously described method (53). All P. aeruginosa strains (PAO1, PAO1 containing plasmid pUCP19, PA14, LESB58, the PAO1ΔPA5441::Gm’ mutant, and the PA14 mutants PA14sad-160 [with a Tn5 insertion between rocA and rocR (sadA and sadR)] that overexpresses sadR rocR), PA14sad-168 [PA0267], PA14sad-199 [sadB, PA5346], and PA14sad-210 mutants) were grown in LBGM30-sucrose plates and 50 ml of brain heart infusion in 250-ml Erlenmeyer flasks. After overnight incubation in a shaking incubator at 37°C, the optical density at 600 nm of each culture was noted. A 200-ml inoculum in 250-ml Erlenmeyer flasks. After overnight incubation in a shaking incubator at 37°C, the optical density at 600 nm of each culture was noted. A 200-ml inoculum in 250-ml Erlenmeyer flasks. After overnight incubation in a shaking incubator at 37°C, the optical density at 600 nm of each culture was noted. A 200-ml inoculum in 250-ml Erlenmeyer flasks. After overnight incubation in a shaking incubator at 37°C, the optical density at 600 nm of each culture was noted.

**Preparation of bacteria for in vivo experiments.** (ii) Construction of PA14 sad mutants. From the collection of random transposon mutants of P. aeruginosa PA14 generated with the transposon Tn5-B21 or Tn5-B20 (Tc) (38, 48), four sad mutants were used for the CI experiments in a rat lung infection model: the PA14sad-160, PA14sad-168, PA14sad-199, and PA14sad-210 mutants. A sadA::Gm’ cassette was modified from a previously described method (53). For construction of the deletion mutant P. aeruginosa PAO1ΔPA5441::Gm’, a previously described strategy was used (13). Briefly, in the first round of PCR, the Gm resistance gene cassette was amplified using the Gm-F and Gm-R primers (Table 3). The 5’ and 3’ fragments of the PA5441 gene were amplified in two PCRs. The first reaction was done with the PA5441-Upf-GWL and PA441-Upf-Gm primers for the constructed deletion of PA5441 and the second reaction with the PA5441-DnF-Gm and PA5441-DnR-GW primers (Table 3). In the second round of PCR, PCR mixture contained the same components as for 5’ and 3’ fragment PCR amplifications, 50 ng of each PA5441 in 5’ and 3’ purified template DNAs, and 50 ng of FRT-Gm-FRT template DNA prepared during the first-round PCR. The BP and LR clonase reactions for recombinatorial transfer of the PCR product into pDONR221 and pEX18ApGw, respectively, were performed as described in Invitrogen’s Gateway cloning manual but with only half of the recommended amounts of BP and LR clonase mixes and E. coli One Shot Max Efficiency DH5α-T1. Transfer of the plasmid (pEX18ApGw)-borne deletion mutations to the P. aeruginosa chromosome was done by electroporation (12). A few colonies were patched on LB-Gm30 plates and LB-Ch200 plates to differentiate single- from double-crossover events. To ascertain resolution of merodiploids, Gm’ colonies were struck for single colonies on LB-Gm30 plates containing 5% sucrose. Gm’ colonies from the LB-Gm-sucrose plates were patched onto LB-Gm30 plus 5% sucrose, as well as LB-Ch200. Colonies growing on the LB-Gm-sucrose plates but not on the LB-Ch200 plates were considered putative deletion mutants. The presence of the correct mutations was verified by colony PCR with the PA5441-Upf-GWL and PA5441-DnR-GW primers (Table 3).

**Preparation of bacteria for in vivo experiments.** (i) Preparation of agarose-embedded bacteria for determination of individual kinetics and CIs. Preparation of the individual P. aeruginosa strains or wild-type–mutant mixtures in agarose beads was modified from a previously described method (53). All P. aeruginosa strains (PAO1, PAO1 containing plasmid pUCP19, PA14, LESB58, the PAO1ΔPA5441::Gm’ mutant, and the PA14 mutants PA14sad-160 [with a Tn5 insertion between rocA and rocR (sadA and sadR)] that overexpresses sadR rocR), PA14sad-168 [PA0267], PA14sad-199 [sadB, PA5346], and PA14sad-210 mutants) were grown in 50 ml of brain heart infusion in 250-ml Erlenmeyer flasks. After overnight incubation in a shaking incubator at 37°C, the optical density at 600 nm of each culture was noted. A 200- to 500-ml aliquot of overnight cultures of single strains or equal ratios of wild-type–mutant mixtures were completed to 5 ml with fresh brain heart infusion to give a final concentration of approximately 1 x 10^8 CFU/100 μl injection.

### TABLE 1. Comparison of the three P. aeruginosa strains used in this study

| Strain      | Approx genome size (Mb) | Pili         | Flagella                      | Genetic elements |
|-------------|-------------------------|--------------|-------------------------------|------------------|
| PAO1 (52)   | 6.264                   | Type IVa class (31) | Highly conserved b-type       | GI (4 ORFs between flgL and flbC) (54) |
| PA14 (33, 44) | 6.537                  | Type IVb class (11, 23) | Highly conserved b-type (54)  | PAPI-1, PAPI-2 (23); GI identical to PAO1 (54) |
| LESB58 (9)  | 6.599                   | ND           | Highly conserved b-type (46)  | PAGI-1, homologous O6 serotype, exoX and type III ploymeride receptor, quorum sensing overexpression (40); PAGI-2 (50) |

a Reference numbers are in parentheses.

b GI, glycosylation island; PAGI-1 and -2, P. aeruginosa genomic islands 1 and 2; PAPI-1 and -2, P. aeruginosa pathogenicity islands 1 and 2.

c ND, not determined.
TABLE 2. Bacterial strains and plasmids used in this study

| Bacterial strain or plasmid | Relevant characteristic(s) or genotype | Reference or source |
|-----------------------------|----------------------------------------|--------------------|
| E. coli ElectroMaxDH10B      | Electrocompetent cells, F' merA Δ(mrr-hsdRMs-mcrBC) &80 lacZΔM15 ΔcatX74 recA endA1 araD139 Δ ara leu7697 galU galK rpsL supG | Invitrogen         |
| One Shot MAX Efficiency     | F' &80 lacZΔM15 Δ(lacZYA-argF)U169 deor recA endA1 hsdR17 (rK mK) pAO1 supE44 thi-1 gyrA96 relA1 tonA | Invitrogen         |

P. aeruginosa

| Strain | Relevant characteristic(s) or genotype | Reference or source |
|--------|----------------------------------------|--------------------|
| PA01   | PAO1293, Cm', E79 ts-2, wild type, derivative of prototrophic PA01 | 28                 |
| PAO1ΔPA5441::Gm' | PAO1293ΔPA5441::Gm', Gm', 934-bp replacement of PA5441 gene with Gm' cassette | This study         |
| PA14   | Wild type, UCBBP-PA14, human isolate   | 33, 44             |
| PA14sad-160 | PA14 sadR'S::Gm', Gm', biofilm mutant, Tn5 insertion between roca and rocR (sadA and sadR) | 29                 |
| PA14sad-168 | PA14sad-168 (PA0267::Tn5B21, Tc', biofilm mutant | 38                 |
| PA14sad-199 | PA14sadB-199 (PAO5346::Tn5B21, Tc', biofilm mutant | 7                  |
| PA14sad-210 | PA14sad-210 (PAO4953, mobI):Tn5B21, Tc', biofilm mutant | 38                 |
| LESB58 | CF isolate, β-lactam resistant, Gm' Az' Im' (Imipenem) | 9, 50              |

Plasmids

| Plasmid | Relevant sequence | Reference or source |
|---------|------------------|--------------------|
| pUCP19  |                   |                    |
| pPS856  |                   |                    |
| pDONR221| Km' Cm' | Gateway pDONR vector with pUC origin, T7 promoter/priming site, M13 forward (−20) and reverse priming sites; rmbB T1 and T2 transcription terminators, attP1 and attR2 sites, ccdB gene | Invitrogen         |
| pEX18ApGW |                   |                    |

TABLE 3. Primers used in this work

| Primer | Relevant sequence | Reference or source |
|--------|------------------|--------------------|
| Gm-F   | GCAATTAGTCTCAAAAGCCGCTTGA |                    |
| Gm-R   | CGAATTTGCGATCTGGAAGTTCTT |                    |
| GW-attB1 | GGCGCAATCGTTTACACAAGGCAGCCGCT |                    |
| GW-attB2 | GGGGACACCTTGGATACTGGGT |                    |
| PA5441-UpF-GWL | TACAAAGAACGCGCTGagggctggaggaaggctggggctgg |                    |
| PA5441-UpR-Gm | TCGAGGCGCTTGGATACTGGT |                    |
| PA5441-DnF-Gm | AGGAAATCTCAAGATCCAAACCATGCGC |                    |
| PA5441-DnR-GW | TTCAGAAAGAACGCGCTTGGT |                    |

a Sequences in uppercase letters are common for all genes to be replaced and overlap with the Gm or attB primer sequence. Lowercase letters indicate PA5441-specific sequences.
sacrificed rats, and homogenized tissues were plated in triplicates on appropriate media.

(i) Infection kinetics of *P. aeruginosa* PAO1, PA14, and LESB58 strains in the rat lung and formaldehyde lung fixation. Thirty-two rats were infected with 120 µl of each agarose-embedded bacterial strain, and eight rats from each group were sacrificed at 1, 3, 7, and 14 days postinfection. From these eight rats, five were sacrificed using an excessive dose of Isoflurane (Baxter) and were used for CFU counts. The three remaining rats were anesthetized using 40 mg ketamine (Bioniche)/kg of body weight and 5 mg/kg xylazine (Novopharm) and were processed for formaldehyde lung fixation. After the thorax was opened, a perfusion needle (no. 22) was used to penetrate the right ventricle of the heart toward the pulmonary artery and was fixed with hemostatic clamps. The left atrium was opened to allow fluids to escape from the system during perfusion. Using a peristaltic pump, approximately 40 ml of 1× PBS solution was administered for 2 min, and then 180 ml of 4% formaldehyde solution in PBS was used for 10 min to fix the rat lung at flow rate of 18 ml/min. The lung was removed very gently to avoid tissue damage and was fixed in the same solution of formaldehyde for at least 24 h. Finally, the lung tissue was embedded in paraffin. Longitudinal sections of 5 µm, collected at regular intervals, were obtained with a microtome from the proximal, medial, and distal lung regions. Sections were stained with hematoxylin-eosin (HE) or with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and used for immunofluorescence (see “Immunolocalization of *P. aeruginosa* in the rat lung” below).

(ii) In vivo CIs. The in vivo CIs were determined for the PAO1/pUCP19-PA14, PAO1/pUCP19-LESB58, and PA14-LESB58 pairs and for the PAO1/pUCP19-PAO1ΔPAS441::Gm’, PA14-PA14sad-166, PA14-PA14sad-168, PA14-PA14sad-199, and PA14-PA14sad-210 wild-type-mutant strain pairs. Injections of approximately 120 µl of each bacterial mixture were administered to ~10 animals. After 7 days of infection, the bacterial counts were performed on infected rat lungs, using PIA for total bacterial number of *P. aeruginosa*; MHA-Ch200 for PAO1/pUCP19 wild-type strain selection; MHA-Gm15 for LESB58, PAO1ΔPAS441::Gm’, or PA14sad-160 mutant selection; and MHA-Tc50 for PA14sad-168, PA14sad-199, or PA14sad-210 mutant strain selection. In preliminary experiments and to confirm that the plasmid is not cured during the in vivo passage, we determined that there were similar numbers of CFU in animals harboring the PAO1 strain with pUCP19 using MHA without and with Ch200 (data not shown). The CI is defined as the CFU output (in vivo) ratio of the mutant in comparison to wild-type strain divided by the CFU input ratio of mutant to wild-type (2, 22). The final CIs were calculated as the geometric mean for animals in the same group.

Immunolocalization of *P. aeruginosa* in the rat lung. Deparaffinized sections of rat lung tissue were analyzed by indirect immunofluorescence using a rabbit antiserum specific for *P. aeruginosa* (kindly provided by J. Pier, Harvard Medical School, Boston, MA). The secondary antibody was Texas Red-labeled goat anti-rabbit immunoglobulin G (Molecular Probes). The slides were examined using an Axiosplan fluorescence microscope (Zeiss), and images were taken with a KS 300 imaging system (Kontron).

**RESULTS**

In vivo growth of *P. aeruginosa* strains in the rat lung. To compare the capacities of the strains to initiate and establish a chronic lung infection in vivo, bacterial growth was monitored by determining CFU from lung tissues at specific time points from day 1 up to day 14 postinfection. As depicted in Fig. 1, the overall growth curves were similar for the three strains tested, with a peak of CFU at day 1 and a reduction in CFU from day 3 to day 7. A plateau was reached at day 7, and there were fewer variations in CFU from day 7 up to day 14. For PAO1 (Fig. 1A) and LESB58 (Fig. 1C), similar numbers of CFU were obtained, where the number of bacteria increased from 1 × 10^6 CFU/lung at injection to 1 × 10^7 CFU/lung at day 1 postinfection. At day 7, we noted a decrease to 8 × 10^6 CFU/lung. This average of CFU was maintained up to day 14. For PA14 (Fig. 1B), the peak of infection appeared at day 3 with 3 × 10^6 CFU/lung, and bacterial counts decreased to the same level as

FIG. 1. In vivo growth curves for *P. aeruginosa* strains PAO1 (A), PA14 (B), and LESB58 (C) in the rat model of chronic lung infection for 14 days. Rates were infected with agarose-embedded bacteria at 1 × 10^6 CFU for each strain. At different time points (1, 3, 7, and 14 days postinfection), five animals were used from each group and CFU were determined from infected lungs.
for the two other strains at day 14. In general, we observed significantly lower bacterial counts for PA14, except at day 3. For LESB58, CFU were higher at day 3 but lower at 7 and 14 days postinfection. These results showed that different *P. aeruginosa* strains were able to initiate and maintain an infection in the rat lung.

**Localization of *P. aeruginosa* in the rat lung.** The localization of bacterial cells and the lung inflammatory response to infection were characterized from the initial challenge at day 1 up to day 14. At days 1 and 3, HE staining and indirect immunofluorescence showed that PAO1, PA14, and LESB58 bacteria were present within beads in the bronchial lumen where they were deposited and induced an intense inflammatory response (data not shown). Analysis at day 7 showed PAO1 and PA14 cells in the alveolar region, where they can form biofilm/macrocultures with extensive inflammation in submucosa and alveoli (Fig. 2D). In contrast, LESB58 bacterial cells were still present in the bronchial lumen (Fig. 2L). Although the chronic lung infection was established using equal CFU, bacterial cells from these three strains were not found with the same localization when the chronic infection was established.

**In vivo competitive analysis of the PAO1, PA14, and LESB58 strains.** To assess the virulence of the three *P. aeruginosa* strains in the rat model of chronic lung infection, we decided to analyze the in vivo competitive growth between strains PAO1-PA14, PAO1-LESB58, and LESB58-PA14. Equal ratios of each strain were mixed in agar beads, the mixture was inoculated into the rat lung, and bacteria were enumerated from the lungs at day 7 postinfection. The CI was calculated, and results are shown in Fig. 3. The competitive analysis between PAO1

![FIG. 2. Localization and persistence of PAO1, PA14, and LESB58 in the rat lung at 7 days postinfection. Rats were infected with *P. aeruginosa* strains embedded in agarose beads, the lungs were fixed and investigated histologically, and bacteria were localized by indirect immunofluorescence. (A, E, and I) HE-stained rat lung histology at 7 days after infection with agarose-embedded PAO1 (A), PA14 (E), and LESB58 (I). Inflammatory cell infiltrations are evident in the thickened alveolar septa of rat lung for the PAO1 and PA14 strains, while for the lungs infected with LESB58, the recruitment of neutrophils is predominantly in the bronchial lumen, where the beads are still localized. (C, G, and K) At day 7, *P. aeruginosa* bacterial macrocolonies were detected by indirect immunofluorescence (IF) (red) in the thickened alveolar septa of rat lungs infected with strains PAO1 (C) and PA14 (G), while for LESB58, (K) bacterial colonies were still present in the agar beads. (B, F, and J) DAPI (blue) staining of the same tissue sections. (D, H, and L) Merge of the DAPI-stained slides (blue) and bacteria localized by IF (red). Bars, 50 μm.**

![FIG. 3. CI analysis of *P. aeruginosa* wild-type strains PAO1, PA14, and LESB58. Each circle represents the CI for a single animal in each group. A CI of less than 1 indicates a virulence defect. The geometric mean of the CIs for all rats is shown as a solid line. CIs for PAO1-PA14 and PAO1-LESB58 were significantly different (P < 0.01).]
and PA14 showed a large variation in lung CFU between each animal and a mean CI value of 0.002. This result indicated a 1,000-fold reduction of PA14 in vivo when in competition with PAO1. A mean CI value of 0.0002 was obtained for the CI analysis between PAO1 and LESB58, suggesting a 10,000-fold attenuation of LESB58 in competition with PAO1. The CI analysis between LESB58 and PA14 gave a mean CI of 0.14, suggesting a 10-fold attenuation of PA14 by LESB58.

Validation of the P. aeruginosa chronic lung infection model using PAO1 and PA14 mutants. (i) Construction of the PAO1ΔPA5441::Gmr’s knockout mutant. The P. aeruginosa STM5441 mutation, inactivating the PA5441 gene, which is part of the two-gene operon PA5441 and PA5442, was identified as attenuated in vivo in the 72 mutant pools by using signature-tagged mutagenesis (42). The PA5441 ORF encodes a putative outer membrane hypothetical protein of 80 kDa. A comprehensive analysis of P. aeruginosa genes encoding the enzymes of cyclic-di-GMP metabolism (diguanylate cyclase [DGC]- and phosphodiesterase [PDE]-encoding genes) was carried out to analyze the function of cyclic-di-GMP in two disease-related phenomena, cytotoxicity and biofilm formation (30). Analysis of the phenotypes of DGC and PDE mutants, including PA5442 mutants and overexpressing clones, revealed that certain virulence-associated traits such as CHO cytotoxicity and biofilm formation are controlled by multiple DGCs and PDEs through alterations in cyclic-di-GMP levels (30). Thus, the intracellular signaling molecule cyclic-di-GMP encoded by PA5442 has been shown to influence bacterial behaviors, including motility, biofilm formation, and cell toxicity. Since the PA5441-PA5442 operon is potentially important and since it had been already selected as attenuated in vivo in the previous study, we decided to use it as a control for our CI experiments in vivo.

To obtain a clean genetic background, the PAO1ΔPA5441::Gmr deletion mutant strain was constructed (13). The 934-bp ΔPA5441 deletion was confirmed by PCR (data not shown). The P. aeruginosa knockout mutant PAO1ΔPA5441 was used for in vivo CI experiments in competition with the wild-type bacteria and was tested for swimming and twitching motility and biofilm formation.

(ii) Identification and characterization of PA14 sad mutants. Four PA14 mutants defective in biofilm production were designated as surface attachment defective (sad mutants). Since biofilm formation is considered an important virulence factor in vivo, the PA14 sad mutants were chosen as controls with the PAO1ΔPA5441 mutant to validate in vivo and in vitro assays.

The DNA sequences flanking the Tn5 insertions in sad mutants were determined using the arbitrary PCR method, and the sequences obtained were compared to the GenBank and P. aeruginosa PAO1 genome sequence (www.pseudomonas.com) databases using BLASTX (38).

The genomic DNA flanking the sad-160::Tn5 transposon insertion indicated that the transposon is located into an intergenic region between two divergently transcribed genes (PA3947 [sadR] and PA3948 [sadA]) and adjacent to the PA3946 (sadS) regulator gene. PA3947 and PA3948 encode proteins homologous to response regulators involved in two-component regulatory systems, and the PA3946 ORF is homologous to sensor histidine kinases. Thus, this locus was referred as a three-component system. Nonpolar mutations in any of the sadARS genes resulted in biofilms with an altered mature structure but did not confer significant defects in growth or early biofilm formation, swimming, or twitching motility. This suggested that the sadARS three-component system is required for later events in biofilm formation on an abiotic surface (29).

PA14sad-168 is a strain that was reconstructed via phage-mediated transduction. The insertion in PA14sad-168 is in the PAO267 gene. This is a gene of unknown function with homology to cheY.

The transposon insertion carried by the PA14sadB-199 mutant was mapped into PA5346, and the ORF encodes a protein of unknown function associated with the biofilm-defective phenotype. Examination of flow cell-grown biofilms showed that the PA14sadB-199 mutant could initiate surface attachment but failed to form microcolonies, despite being proficient in both twitching and swimming motility (7).

The transposon insertion carried by the PA14sadC-210 mutant was mapped to the chemotaxis protein MotB homolog of P. aeruginosa. PA4953 (motB) encodes MotB, a flagellar motor protein involved in cell motility and secretion, and is expressed with motA. RpmA is one of two MotA paralogs in P. aeruginosa, and RpmA and RpmB have been shown to be required for the efficient ingestion of P. aeruginosa by macrophages (49).

(iii) Attenuation of P. aeruginosa PAO1 and PA14 mutants in vivo. To determine the capacity of strains to cause chronic lung infection, one PAO1 mutant strain and four PA14 mutant strains were analyzed using the CI. As depicted in Fig. 4, the mutation in PA5441 caused a defect in vivo maintenance.
After 7 days postinfection, the PAO1/H9004 PA5441::Gmr mutant had a 10-fold decrease of CFU in the rat lung in comparison with the wild-type PAO1, giving an average CI of 0.1, compared to 1.0 for the wild-type PAO1. All four PA14 sad mutants were severely attenuated after 7 days postinfection (from 10-fold down to 1,000-fold), giving average CI values of 0.004, 0.003, 0.05, and 0.009 for PA14sad-160, PA14sad-168, PA14sad-199, and PA14sad-210, respectively.

Phenotypic characterization of the strains. (i) *P. aeruginosa* strain LESB58 demonstrates differences in biofilm formation. Since differences in in vivo virulence and bacterial localization were identified between PAO1, PA14, and LESB58, we tested these strains for biofilm production in microtiter plates of polyvinylchloride (PVC). In this in vitro system, the PAO1 and PA14 strains produced similar biofilm levels (Fig. 5A). Even though LESB58 showed a lower growth rate in minimal medium after 6 h (data not shown), significantly greater amounts of biofilm were produced (Fig. 5A). Compared with the two other strains, the crystal violet-stained ring formed on the walls of PVC wells at the liquid-air interface was thicker for LESB58, and a kind of deposit was observed at the bottom of the well with LESB58 only.

(ii) Reduced biofilm formation for *P. aeruginosa* PAO1 and PA14 mutants. The *P. aeruginosa* PAO1ΔPA5441::Gmr and PA14sad-160, PA14sad-168, PA14sad-199, and PA14sad-210 mutant strains were tested for their ability to form a biofilm. The surface-attachment defective (sad) PA14 mutants were used as negative controls. The PAO1ΔPA5441::Gmr mutant was also defective in biofilm formation; the crystal violet-stained ring formed on walls of PVC wells was smaller than that with the wild-type strain (data not shown). As depicted in Fig. 5A, the quantity of biofilm production indicated less biofilm biomass for the PAO1ΔPA5441::Gmr mutant. The four PA14 sad mutants were defective in biofilm production compared with the wild-type strain PA14.

(iii) Analysis of bacterial motility. Since variations in biofilm production may be caused by changes in flagellum and type IV pilus production (38), we measured the swimming and twitching motilities of the studied strains. Swimming depends upon flagella, whereas twitching depends on type IV pili (25). As shown in Fig. 5B, differences in swimming ability were apparent for the LESB58 wild-type strain, for the ΔPA5441::Gmr deletion mutant, and for the PA14sad-210 mutant. We also assayed the type IV pilus-mediated twitching motility for all
strains (Fig. 4C). Twitching motility was reduced in strain LESB58 only. No correlation between the motility phenotypes and the ability to produce biofilm was apparent in any of the strains tested.

**DISCUSSION**

In this study, we evaluated the kinetics and growth rate in the rat lung infection model for three widely used *P. aeruginosa* strains, PAO1, PA14, and LESB58, using low-melting-point agarose beads. Although there is some evidence that bacteria can escape the agarose beads in vitro, there was no clear evidence that this occurs in vivo. The three *P. aeruginosa* strains studied have similar lung colonization abilities with similar in vivo growth rates in the rat model of chronic lung infection. As shown here, the bacterial release from agarose beads was similar for two prototype strains, whereas bacterial cells of LESB58 remained in agarose beads even after 14 days. Swimming and twitching motilities were reduced in LESB58 in vitro, which could explain maintenance in agar beads.

Using a rat model of chronic lung infection with bacteria embedded in agarose beads, similar infection patterns were clearly displayed for all three strains. At days 1 and 3 some differences were observed between strains. At days 7 and 14 postinfection, chronic lung infection was well established with differences between strains. At days 7 and 14 some differences were observed between strains. At days 7 and 14 postinfection, chronic lung infection was well established with differences between strains. At days 7 and 14 some differences were observed between strains. At days 7 and 14 postinfection, chronic lung infection was well established with differences between strains.

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microarray revealed a conserved pattern of a core genome assembled as a mosaic in many strains. Even if the subsets of 38 gene islands were absent or divergent, no specific pattern was associated with strains isolated from the airways of CF patients (19).

Additional evidence of horizontal gene transfer is the increase in genome size. It was shown that many CF isolates had genomes much larger than the typical 6.3 Mb for PAO1 (24). The genome sizes of some CF isolates ranged from 0.4 to 18.9% larger than that of PAO1 (24). However, it has been shown recently that although extensive novel sequence are present in the genomes of CF isolates, the backbone of the PAO1 genome is still preserved (32, 51).

The in vivo CFU and infection kinetics data presented here suggest that all three strains remain excellent for studying virulence, even though their genome size varies and is not strictly representative of most CF isolates. There is evidence that bacteria in biofilms have an increased incidence of horizontal gene transfer, analogous to the situation observed in hypervirulent LES (46). CI analysis has revealed interesting features of PA01, PA14, and LESB58. Much research on CF has focused on how chronic infection affects the patient because inflammation from infecting bacteria causes persistent respiratory symptoms and an inexorable decline in lung function. However, the onset of chronic infection is also transformative for the bacteria, because an environmental change from infecting bacteria is an excellent example of a transition from reversible to irreversible attachment during biofilm formation by Pseudomonas aeruginosa strain PA14. J. Bacteriol. 186:4746–4755.

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