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Drosophila melanogaster-Based Screening for Multihost Virulence Factors of *Pseudomonas aeruginosa* PA14 and Identification of a Virulence-Attenuating Factor, HudA▽†

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***Pseudomonas aeruginosa* is an important opportunistic human pathogen that interacts with phylogenetically diverse nonmammalian hosts, including plants, nematodes, and insects. Here, we exploited the *P. aeruginosa*-induced killing of the fruit fly *Drosophila melanogaster* as an assay system to screen for virulence-attenuated mutants of *P. aeruginosa* PA14. Fifteen nonredundant mutants were isolated from 4,018 random transposon (Tn*phoA*) insertion clones, and 13 out of them (86.7%) displayed significantly reduced virulence in a murine peritonitis model as well. The Tn*phoA* insertion sites of the 15 mutants were determined; already known virulence genes (*dsbA*, *pvdI*, *fhlB*, *pilF*, and *wspF*) and new virulence genes such as PA0253 (*hudR*), PA0369, PA2077, PA0272, PA2113, PA2965 (*fabF1*), and PA2002 were identified; one insertion was located at the intergenic region between PA1928 and PA1929; and the other two insertions were located in the genes (PA14_35740 and PA14_36000) within a putative genomic island, indicating a potential pathogenicity island of PA14. Further characterization of *hudR*, a virulence gene which encodes a MarR/SlyA family transcription factor, revealed that elevated expression of PA0254 (*hudA* [*h*omologous to *U*bi*D*]) was necessary and sufficient for the virulence attenuation of the *hudR* mutant. The HudR protein repressed the *hudAR* operon by directly binding to its upstream promoter region. Collectively, these results validate the relevance of the *D. melanogaster* model for the high-throughput identification of new virulence factors involved in the multihost pathogenesis of *P. aeruginosa*.**

Pseudomonas aeruginosa is a common opportunistic human pathogen that is responsible for fatal infections in immunocompromised individuals, such as hospitalized patients and those suffering from severe burns or other traumatic skin damage or from cystic fibrosis. This ubiquitous gammaproteobacterium deploys an arsenal of diverse virulence factors to infect hosts of multiple phylogenetic backgrounds that include vertebrates, plants, insects, and nematodes. Surprisingly, many studies reveal the extensive conservation of the virulence mechanisms exploited by *P. aeruginosa* to intoxicate such evolutionarily divergent hosts (28). Some virulence factors are commonly required for those hosts and have been newly identified from screening using nonmammalian model hosts, such as plants (34) and *Caenorhabditis elegans* (37). Since mammalian host models for studying host-pathogen interactions have some limitations in terms of genetic unwieldiness, cost-effectiveness, and ethical restraints, the idea of using genetically tractable nonmammalian host organisms is attractive, based on the pathogenic promiscuity with considerable conservation of virulence mechanisms.

Among the nonmammalian model hosts, the fruit fly *Drosophila melanogaster* is genetically well defined and possesses a well-characterized innate immune system to defend against microbial pathogens (17). In *D. melanogaster*, the activation of the immune response is regulated primarily by two parallel signaling pathways involving Toll and Imd (25), since these pathways regulate almost 80% of the genes induced upon septic injury (8).

Based on the molecular similarities to humans in innate immune signaling as well as the genetic tractability, much attention has been paid to *D. melanogaster* as an alternative invertebrate animal host to model the human-pathogen interaction. *D. melanogaster* pathogenesis models have been established for diverse bacterial pathogens that include *P. aeruginosa*, *Vibrio cholerae*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Mycobacterium marinum* (7, 9, 22, 31, 32, 33). In those models, the disease symptom is a systemic spread of the infecting bacteria in general, which leads to bacterial proliferation and fly mortality, although the killing kinetics and mortalities differ. A screen using *D. melanogaster* to identify virulence-attenuated mutants of the *P. aeruginosa* PAO1 strain was previously performed, and a subset of virulence genes were identified, most of which are associated with twitching motility (7). This and the finding that the immune signaling pathway involving the Toll and Imd pathways is important in resistance to *P. aeruginosa* infections (22) corroborate the use of *D. melanogaster* for high-throughput screening of *P. aeruginosa* virulence factors potentially involved in the complex interactions with host immunity factors which underlie human diseases caused by *P. aeruginosa* infection. By means of optimizing the infection condition for the primary screening, we here isolated a largely distinct set of virulence-attenuated mutants from *P. aeruginosa* strain PA14, most of which are also important in a mouse model as well.

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TABLE 1. *P. aeruginosa* PA14 mutants and plasmid constructs used in this study

Mutant or construct	Relevant characteristic(s)[a]	Reference
Tn*phoA* insertion mutants		
102G4	Tn*phoA* insertion in *dsbA*; Km^r	This study
113A8	Tn*phoA* insertion in PA14_36000; Km^r	This study
122G3	Tn*phoA* insertion in PA0272; Km^r	This study
124E2	Tn*phoA* insertion in *pilF*; Km^r	This study
133B5	Tn*phoA* insertion in *flhB*; Km^r	This study
152A9	Tn*phoA* insertion in *hudR*; Km^r	This study
152B8	Tn*phoA* insertion in *hudR*; Km^r	This study
152F1	Tn*phoA* insertion in PA2113; Km^r	This study
153B10	Tn*phoA* insertion in PA2002; Km^r	This study
153C4	Tn*phoA* insertion in PA0369; Km^r	This study
161B2	Tn*phoA* insertion in PA14_35740; Km^r	This study
162C1	Tn*phoA* insertion in PA2424; Km^r	This study
162C3	Tn*phoA* insertion between PA1928 and PA1929; Km^r	This study
162E4	Tn*phoA* insertion in PA2424; Km^r	This study
165C11	Tn*phoA* insertion in *wspF*; Km^r	This study
167B1	Tn*phoA* insertion in *fabF1*; Km^r	This study
In-frame deletion and related mutants		
hudR	In-frame deletion of *hudR*	This study
hudA	In-frame deletion of *hudA*	This study
hudAR	In-frame deletions of *hudA* and *hudR*	This study
Plasmid constructs		
pUCP-HudR	pUCP18 with the 1.2 kb of the *hudR* gene; Cb^r	This study
pUCP-HudA	pUCP18 with the 2.0 kb of the *hudA* gene; Cb^r	This study
pUCP-HudAR	pUCP18 with the 3.0 kb of the *hudA* and *hudR* genes; Cb^r	This study
pQF-hudAp	pQF50 with the 466 bp fragment of the *hudA* upstream; Cb^r	This study

[a] Cb^r, carbenicillin resistant.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *Escherichia coli* strains DH5α, BL21(DE3)pLysS, and S17-1, for general-purpose cloning, protein overexpression, and conjugal DNA transfer, respectively, and the wild-type *Pseudomonas aeruginosa* strain PA14 and its derivates listed in Table 1 were used in this study. All strains were grown overnight (for 14 to 18 h) at 37°C using Luria-Bertani (LB) broth and M63-citrate minimal medium [1.2% NH_2PO_4, 2.8% K_2HPO_4, 0.8% $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 4% citrate] or on 2% Bacto agar (Difco) LB or cetrimide agar (Difco) plates as described previously (15). Overnight cultures were inoculated into the fresh LB broth with an inoculum size of 1.6×10^7 CFU/ml, grown at 37°C for 3 to 5 h with agitation to the early stationary phase, and used for experiments.

DNA oligonucleotide primers. The DNA oligonucleotide primers used for gene deletion, gene expression, and gene detection in this study are listed in Table S1 in the supplemental material.

Transposon mutagenesis. Transposon-mediated mutagenesis of PA14 was performed by using plasmid pRT733 carrying Tn*5*-derived transposon Tn*phoA* (30). The recipient PA14 cells and the donor *E. coli* S17-1 pRT733-carrying cells were grown in LB broth for 12 h at 37°C. Donor and recipient cells were plated together on LB agar plates and incubated at 37°C for 20 h, and PA14 cells carrying a chromosomal transposition of Tn*phoA* were selected on LB agar plates containing rifampin (200 μg/ml) (to counterselect the *E. coli* donor cells) and kanamycin (Km) (500 μg/ml) (to select Tn*phoA*-containing PA14 cells). Single colonies were patched for verification of Tn*phoA* transposition by PCR amplification of a 784-bp fragment from the Km marker of Tn*5* by use of Km-F and Km-R primers as described previously (5). To discard the whole plasmid integration, we performed PCR amplification of pRT733 replication origin by use of pUCori-F and pUCori-R, which amplifies a 711-bp fragment from pRT733. The library complexity was roughly estimated based on the arbitrary PCR patterns from 30 randomly chosen transposon clones. A total of 4,018 Tn*phoA*-positive and pRT733 replication origin-negative transposants were subjected to this screen.

***D. melanogaster*-based screening procedure.** For the primary *D. melanogaster*-based screening, each Tn*phoA* insertion clone that had been frozen was carefully inoculated into 96-well plate-based fresh LB broth (150 μl) containing Km (200 μg/ml) at 37°C. Cells were grown for exactly 6 h and directly used for fly infection without dilution. Fly infection was performed by pricking at the dorsal thorax as described elsewhere (23, 24), using *D. melanogaster* Oregon R flies that were grown at 25°C using corn meal-dextrose medium (0.93% agar, 6.24% dry yeast, 4.08% corn meal, 8.62% dextrose, 0.1% methyl paraben, 0.45% [vol/vol] propionic acid). Two groups of six flies were infected with each Tn*phoA* clone. After 40 h postinfection, wild-type PA14 cells displayed 100% mortality, and 54 clones that had allowed more than two survivors in both infections were selected as the primary candidates. The levels of prototrophic growth of the 54 candidates were assessed based on the growth rate in M63-citrate minimal medium, by which six clones were discarded. For the secondary screening, virulence measurement of the 48 clones was performed by infecting groups of 100 flies with bacterial cells as described below to give the 43 positive clones, which were subjected to allelic exchange. The transfer of the mutant allele was verified by PCR detection of the Km marker, and then clones were subjected to virulence measurement using *D. melanogaster*. Only 16 clones were positive at this stage, and these were subjected to Southern hybridization based on SalI digestion.

Virulence measurements. Fly mortality was determined using groups of 100 male flies of the *D. melanogaster* Oregon R strain. Flies were infected with 50 to 200 CFU of bacteria that had been grown in LB broth to an optical density at 600 nm (OD_{600}) of 3.0 and kept at 25°C. Flies that died within 18 h postinfection were excluded from mortality determination. For mouse infection, LB broth-grown bacterial cells (OD_{600} of 3.0) were harvested, washed twice with phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate, pH 7.0), and appropriately diluted to reach either 2×10^6 or 5×10^6 CFU in 100 μl of PBS containing 1% mucin, which helps to induce infection in naive mice as an adjuvant. Groups of 10 anesthetized ICR mice (4 weeks old) were infected intraperitoneally using 2×10^6 or 5×10^6 CFU, depending on the strains included for the result. Mice were regarded as moribund when they exhibited nonresponsiveness to stimuli, ruffled fur, and evidence of dehydration. More than 90% of the mice that had been infected with the wild-type PA14 became moribund within 24 h. Kaplan-Meier analysis and log rank tests were used to compare the virulence difference between the two groups (1). A *P* value less than 0.05 was considered significant.

Allelic exchange. Allelic exchange to transfer the Tn*phoA* insertion region to a new PA14 background was performed according to the work of Choi et al. (4), with slight modification. Cells from overnight, stationary-phase cultures grown in LB were harvested for 2 min at 8,000 rpm. The cell pellet was washed twice with 1 ml of 300 mM sucrose, resuspended in 100 μl of 300 mM sucrose, and then

used for electroporation. Chromosomal DNA samples (500 ng) obtained from the Tn*phoA* transposants were mixed with the electrocompetent *P. aeruginosa* cells. After a pulse (25 μF, 2.5 kV/cm, 5 ms) was applied, 1 ml of prewarmed LB medium was promptly added, and cells were transferred to a glass tube and shaken for 1 h at 37°C for regeneration. Cells were spread on LB plates containing Km (200 μg/ml) and incubated at 37°C until Km-resistant colonies appeared. PCR amplifications of the Km marker as well as the gene of interest verified the expected genetic structure by allelic exchange.

Transposon insertion site determination. Two methods (arbitrary PCR and direct cloning) followed by sequencing were used to determine the transposon insertion sites, essentially as described elsewhere (5).

Generation of in-frame deletion and double mutants. All the in-frame deletion mutants in this study were created using pEX18T (16). The designs of oligonucleotide primers were based on the PA14 genome sequence (NCBI accession numbers AABQ06000000 to AABQ06000008K; T. Montgomery, G. Grills, L. Li, W. A. Brown, J. Decker, R. Elliot, et al., 2002. *Pseudomonas aeruginosa* strain UCBPP-PA14 whole-genome shotgun sequencing project. http://www.ncbi.nlm.nih.gov.). We created *hudR*, *hudA*, and *hudAR* mutants basically by gene splicing by overlap extension (19) using four oligonucleotide primers listed in Table S1 in the supplemental material. In general, more than 30% of the coding regions were deleted for all the deletions, which were verified by PCR.

Episomal gene expression. The HudR and HudA proteins were ectopically expressed using a multicopy plasmid, pUCP18. For HudR, the 1.2-kb DNA fragment encompassing the entire coding region of the *hudR* gene was cloned by HindIII and SphI digestion of the 1.5-kb DNA fragment amplified using hudR-N2 and hudR-C2. Similarly, the 2.0-kb DNA fragment for the *hudA* coding region (with hudA-N2 and hudA-C3) and the 3.0 kb for both *hudA* and *hudR* coding regions (with hudA-N2 and hudR-C2) were appropriately cloned into pUCP18 as pUCP-HudA and pUCP-HudAR, respectively. All the pUCP18-based constructs were introduced into PA14 and its isogenic mutant bacteria by electroporation (4).

Microarray analysis. Affymetrix PAO1 GeneChip was used for microarray analysis. RNA was isolated from cultures at the logarithmic-growth phase (OD_{600} of less than 1.0). Trizol (Invitrogen) was used and the samples were treated according to the manufacturers' recommendation. In addition, we treated eluted RNA with DNase I for 1 h at 37°C. After enzyme inactivation at 65°C for 1 h, further treatment of RNA samples was done as described by the manufacturer (Affymetrix) with minor modifications as suggested by previous studies (38). cDNA synthesis, fragmentation, and labeling and *P. aeruginosa* PAO1 gene chip hybridization and washing were performed according to the instructions of the manufacturer. Three independent experiments were performed using distinct RNA preparations. Data analysis was performed using Affymetrix microarray suite software suite (MAS) (version 5.0) with Affymetrix default parameters. We calculated the change as the ratio between the signal averages of three *hudR* mutant (experimental) and three wild-type (control) cultures. Gene expression changes were identified with statistical significance, based on the Mann-Whitney U test, with a cutoff *P* value of 0.05 (2).

Reporter fusion promoter assay. The 466-bp DNA fragment encompassing the potential *hudAR* promoter (*hudAp*) region, which spans from nucleotide −442 to +24 relative to the initiation codon of *hudA*, was prepared by PCR amplification using hudA-N2 and hudA-C4. The amplified DNA fragment was cloned into pQF50 (10). LacZ (β-galactosidase) activity was measured according to the method of Zubay et al. (40) with slight modifications. Briefly, cells were grown in LB broth from the early log phase to the late stationary phase. At the designated time points, each culture was subjected to OD_{600} measurement, whereas 70 μl of the culture was mixed with 630 μl of Z buffer (1.61% $Na_2HPO_4 \cdot 7H_2O$, 0.55% $NaH_2PO_4 \cdot H_2O$, 0.075% KCl, 0.0246% $MgSO_4 \cdot 7H_2O$, 0.27% [vol/vol] β-mercaptoethanol) and then disrupted by adding 14 μl of chloroform and 14 μl of 0.1% sodium dodecyl sulfate (SDS). The cell extracts were incubated at 30°C for 15 min, and assays were started by adding 140 μl of *O*-nitrophenyl-β-D-galactopyranoside (4 mg/ml). Reactions were stopped by adding 350 μl 1 M Na_2CO_3. The absorbance of reaction mixtures was determined at 420 nm. Miller units were calculated based on the following formula: $1{,}000 \times OD_{420}/[\text{time (min)} \times \text{volume (ml)} \times OD_{600}]$.

Recombinant protein expression. pET15b was used for recombinant HudR (rHudR) protein overexpression. The coding region of the *hudR* gene was amplified using hudR-N0 and hudR-C2. The PCR product following NdeI and HindIII digestion was cloned into pET15b, and then the resultant plasmid was introduced into *E. coli* BL21(DE3)pLysS. *E. coli* cells were grown at 37°C for 2 to 3 h with agitation to the early log phase and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the overproduction of HudR protein was verified by 12% SDS-polyacrylamide gel electrophoresis (PAGE).

Antibody preparation and Western blot analysis. His-tagged rHudR was recovered from the inclusion bodies and then gel purified from a 10% SDS-polyacrylamide gel. An emulsion of 100 μg HudR protein in PBS was injected peritoneally into five mice (ICR females) at an interval of 2 weeks. After 5 weeks, antisera were prepared from the sacrificed mice. For Western blot analysis, a 10% SDS gel was applied for electrotransfer to a polyvinylidene difluoride membrane (Amersham) by use of the Trans-Blot system (Bio-Rad) at 160 mA for 1 h. The membranes were washed three times, blocked in Tris-buffered saline–Triton X-100 (TBS-T; 20 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1% Triton X-100) containing either 0.5% bovine serum albumin or 2% skim milk and incubated for 1 h with anti-catalase antibodies (10^{-4} dilution). Excess antibodies were removed by repeated washing with TBS-T. After 15 min of incubation in TBS-T containing the secondary antibody (10^{-4} dilution of goat immunoglobulin G against mouse immunoglobulins; Santa Cruz) conjugated with horseradish peroxidase, membranes were washed twice with TBS-T. The immunodetection was performed using the ECL detection system according to the manufacturer's instruction (Amersham). Anti-RpoA antibody was used to control the protein amount.

Gel shift assay. A gel mobility shift assay was carried out as described previously (3) using the 466-bp *hudAp* fragment (see Fig. 3A). The labeled probe (about 30,000 cpm for <0.1 pmol per reaction) was incubated with affinity-purified rHudR (10 pmol) and *P. aeruginosa* cell extracts (20 μg) containing HudR by use of 20 μl of binding buffer (4 mM Tris-HCl, pH 8.0, 1 mM EDTA, 4 mM dithiothreitol, 5 mM $MgCl_2$, 20 mM KCl, 0.3 μg/ml bovine serum albumin, 10% glycerol) plus 1 μg of poly(dI-dC) at 25°C for 10 min. The DNA-protein mixture was electrophoresed on a 5% native polyacrylamide gel in 1× Tris-borate-EDTA buffer (35) and was analyzed by a phosphorimage analyzer (Fuji).

RESULTS

Use of *Drosophila melanogaster* to isolate virulence-attenuated mutants of *P. aeruginosa* strain PA14. To identify *P. aeruginosa* virulence-related genes, we devised and optimized a screening procedure to identify bacterial mutants with reduced virulence in the fruit fly *D. melanogaster*. A total of 4,018 PA14 Tn*phoA* insertion mutants were individually tested for attenuated virulence by use of wild-type Oregon R flies as described in Materials and Methods, which yielded 54 candidates for virulence-attenuated mutants after the primary screening. The whole screening procedure is summarized in Fig. 1. To determine whether the observed attenuation in virulence was due to a problem of general metabolism and/or growth or was the result of the disruption of a virulence gene, we next determined basal growth parameters in liquid culture such as doubling time, lag time, and saturation optical density, since mutants that grew slowly or showed auxotrophism might be less virulent in *D. melanogaster*. Six clones were discarded due to the markedly slower growth. The remaining 48 clones did not show any growth defect in M63-citrate minimal medium and were subjected to the secondary screening. As the secondary screening, we tried to confirm the virulence attenuation of the 48 candidates by infecting groups of 100 flies and diluted bacterial cells after adjusting the growth phase and bacterial amount introduced as described in Materials and Methods. We selected the 43 clones from this secondary screening and transferred their mutation loci into a new PA14 background (i.e., allelic exchange) in order to verify whether the virulence-attenuated phenotype is due to Tn*phoA* insertion. As a result, we reduced the number of the positive candidates to 16 by discarding 27 clones at this step. This dramatic reduction of the positive candidates may indicate that secondary mutations were generated during the transposon mutagenesis. Each of the 16 mutant clones contained a single transposon insertion, as verified by Southern blot analyses, whereas two clones (152A9 and 152B8) displayed the same band patterns (data not shown), suggesting

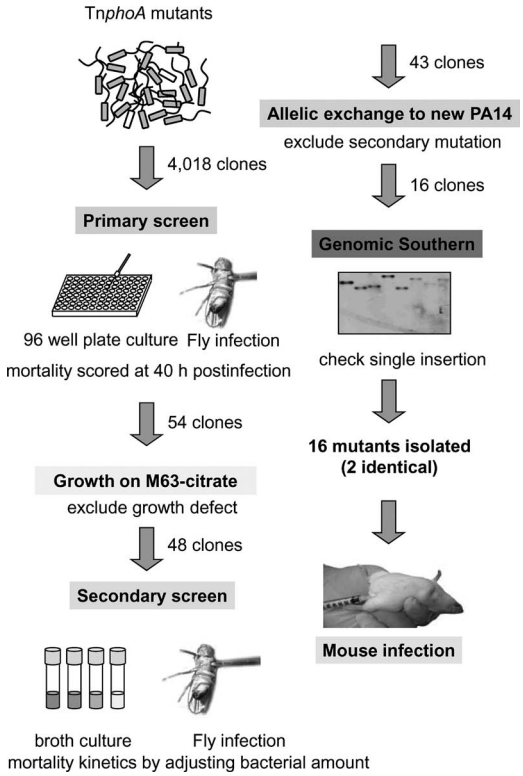

FIG. 1. Schematic drawing of the *Drosophila melanogaster*-based screening procedure. The whole procedure in this study consists of the following six steps, with the remaining number of candidates shown after each step: transposon library construction (4,018 clones), primary screening (54 clones), prototrophic growth verification (48 clones), secondary screening (43 clones), allelic exchange (16 clones), and Southern hybridization (16 clones). The final 16 candidates were tested for virulence attenuation in a mouse peritonitis model (Fig. 2). The details of the screening procedure, including transposition verification and complexity estimation, are described in Materials and Methods.

that these two mutants are likely identical (Table 2). The 16 isolated mutants were classified into the following three categories based on their virulence attenuation in *D. melanogaster*: weakly attenuated (70 to 90%), moderately attenuated (50 to 70%), and highly attenuated (10 to 50%) (Fig. 2A).

Most of the isolated mutants exhibit reduced virulence in a murine infection model. To determine whether the isolated genes required for full virulence in the *D. melanogaster* model were also necessary for pathogenesis in mammalian hosts, the 16 mutants were tested in a mouse infection model. We exploited a murine peritonitis model described elsewhere (24). Fourteen out of the 16 mutants showed a reproducible attenuation of their virulences in this model (Fig. 2B and Table 2). Among them, four mutants (122G3, 152F1, 161B2, and 162E4) showed 100% mortality with significantly delayed killing at 5×10^6 CFU infection (Fig. 2B). The virulences of two mutants (153B10 and 162C3) were not attenuated at all in this mouse model, even though 153B10 is a highly attenuated mutant in *D. melanogaster* (25% mortality) (Fig. 2A). As a whole, the mortalities in *D. melanogaster* and mouse peritonitis models do not display quantitative correlation. Nevertheless, the mutants that we have isolated from this *D. melanogaster* screen are impaired in the genes most likely involved in the multihost pathogenesis of *P. aeruginosa* (Table 2).

Molecular characterization of the virulence-attenuated mutants. To identify the transposon insertion sites of the isolated mutants, we carried out arbitrary PCR and direct cloning as described previously (5), through which we determined the junction region between the transposon and the genomic DNA of each mutant. The gene identities of the isolated 16 mutants are summarized in Table 2. As might be expected from the Southern analysis, 152A9 and 152B8 were identical, and their transposon insertion was mapped within a gene (PA14_03120) encoding a putative transcription factor of yet-unknown function. Two genes, *dsbA* (102G4) and *pvdI* (165C11), have been already known to be necessary for the virulence of *P. aeruginosa* (11, 13). The two corresponding mutants showed highly attenuated virulence in both *D. melanogaster* and mouse models (less than 50%), corroborating the importance of periplasmic disulfide isomerization and pyoverdine synthesis in *P. aeruginosa* multihost pathogenesis. We identified three motility-related mutant genes (*pilF*, *flhB*, and *wspF*) and 10 new virulence genes involved in *D. melanogaster* infection. Among them, eight genes are present on the PAO1 genome as well, representing some conserved functions in *P. aeruginosa* strains, whereas two genes (PA14_36000 and PA14_35740) are absent from PAO1. Among the eight genes, one gene (specific to 162C3) is located at the intergenic region between PA1928 and PA1929. Since we did not perform the complementation for all the isolated mutants, we are still unable to exclude the possibilities of polar effect or others at this stage.

Interestingly, the PA14_36000 and PA14_35740 genes encode a transcriptional regulator and a transposase, respectively, both of which are located within a potential genomic island of about 35 kb of PA14. This genomic island in PA14 seems to have replaced the genomic region corresponding to the region between PA2218 and PA2234 (*pslD*) of the PAO1 genome. This genomic island corresponds to the 13th region from the 24 potential variable regions of PAO1, previously proposed by Wolfgang et al. (38). These results suggest that this genomic island may be a pathogenicity island, and further investigation in terms of the functional and structural aspects of the genes that this island contains is needed.

HudR (PA14_03120) is a transcriptional repressor of the *hudAR* operon. We paid further attention to the PA14_03120 (PA0253) gene, which has been identified from two independent clones in our screen with relatively remarkable virulence attenuation in both *D. melanogaster* (about 65% mortality) and mouse (60% mortality) models. The PA14_03120 gene encodes a potential transcription factor belonging to the MarR/SlyA-family of transcriptional regulators (http://v2.pseudomonas.com). We named this gene *hudR* (see below) and created an in-frame deletion mutant, as schematically represented in Fig. 3A. The virulence attenuation of the *hudR* mutant was almost the same as those of the original Tn*phoA* mutants (152A9 and 152B8) in *D. melanogaster* and mouse infections (Fig. 3B and C). Furthermore, the virulence attenuation phenotype was fully complemented by pUCP18-based expression of the *hudR* gene in the deletion and the insertion mutants. This result suggests that a gene encoding a transcription factor involved in multihost pathogenesis was identified in this screen.

TABLE 2. *P. aeruginosa* PA14 mutants displaying reduced virulence by *Drosophila melanogaster* screening

Mutant ID	Gene ID[a]		Gene name[c]	% Mortality		Description
	PAO1 ID	PA14 ID		Fly[d]	Mouse[e]	
102G4	PA5489	PA14_72450	*dsbA*	27.0***	10***	Periplasmic disulfide isomerase
165C11	PA3703	PA14_16480	*wspF*	70.0*	10***	Methylesterase
162C1	PA2402	PA14_33610	*pvdI*	48.3***	50***	Nonribosomal peptide synthetase
124E2	PA3805	PA14_14850	*pilF*	36.0***	60***	Type IV pilus assembly protein
152A9	PA0253	PA14_03120	*hudR*	64.3**	60**	MarR/SlyA family transcription factor[h]
152B8	PA0253	PA14_03120	*hudR*	66.0**	60**	MarR/SlyA family transcription factor[h]
153C4	PA0369[b]	PA14_04850		72.2**	70**	Hypothetical protein
167B1	PA2077	PA14_37650		69.7**	70**	Hypothetical protein with type I export signal
133B5	PA1449	PA14_45720	*flhB*	23.1***	80***	Flagellar biosynthesis protein
113A8		PA14_36000	*prpR*	46.7***	80**	RocR-type transcription factor[i]
122G3	PA0272	PA14_03530		72.4*	100**[f]	LysR-type transcription factor[j]
152F1	PA2113	PA14_37260	*opdO*	54.0***	100**[f]	Outer membrane pyroglutamate porin
161B2		PA14_35740		57.7***	100*[f]	Transposase
162E4	PA2965	PA14_25690	*fabF1*	33.3***	100**[f]	Beta-ketoacyl-acyl carrier protein synthase II
153B10	PA2002	PA14_38610		25.0***	100[g]	Short chain fatty acid transporter
162C3	IG	IG		84.6*	100[g]	Between PA1928 and PA1929

[a] Gene ID designations respresent the PA number on the PAO1 genome and the PA14 number on the PA14 genome; two genes (113A8 and 161B2) are not present on the PAO1 genome. IG, intergenic region.

[b] The initiation codon of PA0369 (PA14_04850) was reassigned based on a better ribosome binding signature, which changed the protein length (from 99 aa to 188 aa).

[c] Gene names are taken from the current annotation information available at http://v2.pseudomonas.com; PA14_03120 and PA14_03130 are newly named as *hudR* and *hudA* in this study. The transposon insertion positions of the two mutants (152A9 and 152B8) are identical.

[d] Fly mortality was scored after 48 postinfection. The listed mortalities are selected based on the statistical significance ($P < 0.05$) in comparison with the wild type by the Kaplan-Meier log rank test as described for Fig. 2, with P values indicated as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

[e] Mouse mortality was scored after 48 postinfection (infection with 5×10^6 CFU). The statistical significances in comparison with the wild type by the Kaplan-Meier log rank test as described for Fig. 2 are indicated as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

[f] Four mutants (122G3, 152F1, 161B2, and 162E6) showed marked delays in reaching 100% mortality compared to the wild type (Fig. 2B).

[g] Two mutants (153B10 and 162C3) displayed wild-type-level mortalities under our experimental conditions, with P values of 0.561 and 0.187, respectively.

[h] The MarR/SlyA family transcription factor contains a PFAM01047 (MarR-type helix-turn-helix) domain.

[i] The RocR-type transcription factor contains multiple domains such as PFAM00989 (PAS), PFAM00158 (σ^{54} interaction), and PFAM02954 (FIS-type helix-turn-helix).

[j] The LysR-type transcription factor contains both PFAM00126 (LysR-type helix-turn-helix) and PFAM03466 (substrate-binding) domains.

As an initial attempt to decipher the role that HudR plays in the virulence pathways of *P. aeruginosa*, we performed GeneChip analyses using the *hudR* mutant. Table 3 lists the genes that were significantly ($P < 0.05$) changed in the *hudR* mutant compared to the wild type. We found that the adjacent gene (PA14_03130, or PA0254) was upregulated in the *hudR* mutant and verified the higher basal expression by promoter fusion analysis (Fig. 4), which indicates that HudR may act as a repressor of the nearby gene, PA14_03130. Interestingly, PA14_03130 encodes a 496-amino-acid (aa) protein that displays the highest similarity to a hypothetical protein (An09g00030) from *Aspergillus niger* (BLASTP E value = 2.0×10^{-122}) and related hypothetical proteins from various fungal species in the phylum of *Ascomycota* (see Table S2 in the supplemental ma-

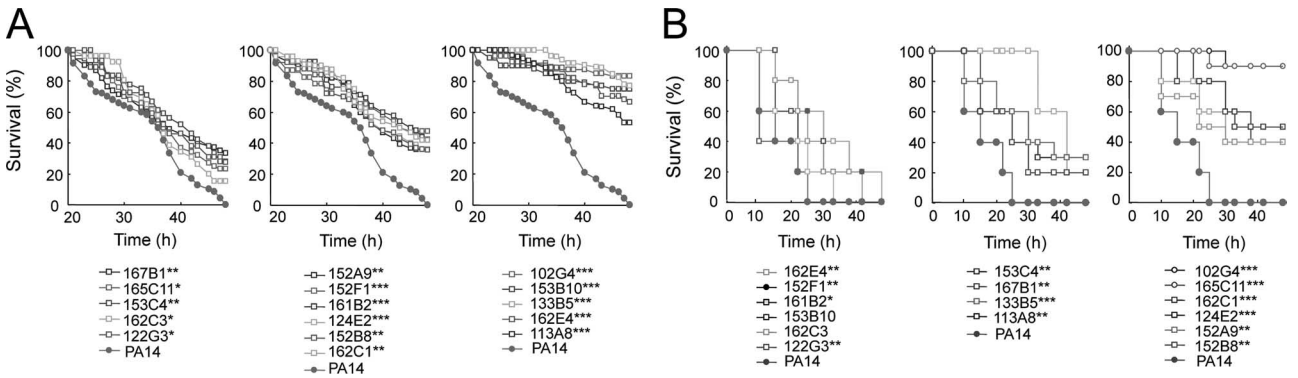

FIG. 2. Virulence measurement for the isolated mutants. The virulences of the isolated mutants were measured based on the mortality curves for *D. melanogaster* (A) and mouse (B) infections. For mouse infection, 5×10^6 bacterial CFU were intraperitoneally administered. In both panels, the mortality curves of the mutants are shown as divided into the following three groups: slightly attenuated (left), moderately attenuated (middle), and highly attenuated (right). Survival was determined following infection by the 16 isolated candidates, as described in Materials and Methods. The values are the averages of five replicate experiments for *D. melanogaster* (A) and three independent experiments for mouse (B) infections. Statistical significance for the virulence difference compared with the wild type is indicated for each mutant (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). The mortality values for each mutant in both models are summarized in Table 2.

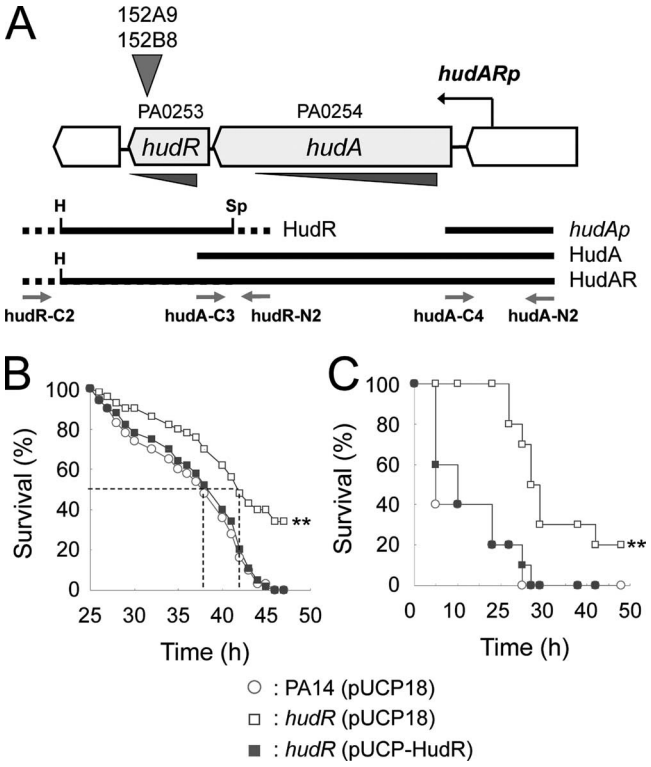

FIG. 3. Virulence attenuation of the *hudR* mutant. (A) Schematic representation of the *hudAR* operon with the regions for deletions (for *hudR*, 402 bp; and for *hudA*, 1,020 bp) and ectopic expressions (for HudR, 1,227 bp; for HudA, 2,034 bp; and for both HudA and HudR [HudAR], 2,974 bp) shown as indicated. The potential promoter element (*hudARp*) and the 466-bp DNA fragment used for the gel shift experiment (Fig. 5) are represented as a bent arrow and a solid line, respectively. The transposon insertion sites (for both 152A9 and 152B8) are indicated by the inverted triangle. The dotted line represents the deleted DNA region by HindIII (H) and SphI (Sp) digestion following PCR amplification. The arrows indicate the positions of the primers (for detailed information, see Table S1 in the supplemental material). (B and C) The virulence of the *hudR* in-frame deletion mutant in *D. melanogaster* (B) and mouse (C) infections. For mouse infection, 5×10^6 bacterial CFU were used. Survival was determined as described in Materials and Methods. The values are the averages of five replicate experiments for *D. melanogaster* (B) and three experiments for mouse (C) infections. The dotted lines in panel B represent the times required to reach 50% mortality. PA14 (pUCP18), PA14 containing pUCP18 plasmid; *hudR* (pUCP18), the *hudR* deletion mutant containing pUCP18 plasmid; *hudR* (pUCP18-HudR), the *hudR* mutant containing pUCP18-based expression constructs of HudR, as indicated in panel A. The statistical significance for the virulence difference compared with the wild type is indicated (**, $P < 0.01$).

terial). *P. aeruginosa* HudA and the fungal proteins contain the signature of UbiD enzyme (PFAM01977), which is 3-polyprenyl-4-hydroxybenzoate decarboxylase, involved in ubiquinone biosynthesis. Since *P. aeruginosa* has the *ubiD* ortholog (PA5237, or PA14_69150) on the genome, we renamed PA14_03130 as *hudA* (*h*omologous to *Ubi*D).

Since both *hudA* and *hudR* genes are separated by only 41 bp, with no potential transcription termination signal therebetween, we verified the operon structure by PCR amplification of the intergenic region from the cDNA generated by reverse transcriptase PCR (data not shown). Furthermore, we did not find any promoter activity of the DNA fragment spanning from the *hudA* initiation codon to the middle of the *hudR* gene (data not shown). Therefore, we concluded that the *hudR* gene is cotranscribed with the *hudA* gene from the *hudAp* (or *hudARp*) promoter.

To monitor the gene expression from the *hudAp* promoter during growth, the *hudAp-lacZ* fusion was constructed as described in Materials and Methods and then introduced into both PA14 and the *hudR* mutant. They exhibited identical growth curves (Fig. 4A). As shown in Fig. 4B, the basal promoter activity was higher in the *hudR* mutant than in PA14. It is noticeable that transcription from the *hudAp* promoter was suddenly increased once the cells entered the stationary growth phase. The stationary-phase increase of the *hudAR* expression is independent of the HudR repressor. These results suggest that the potential role of the *hudAR* operon may be implicated during the stationary growth phase and that the stationary-phase induction of *hudAR* expression requires regulators other than HudR itself.

The operon structure and the involvement of the HudR repressor in the basal expression of the *hudAR* gene led us to examine the possibility of direct regulation by HudR, which would constitute an autoregulatory loop. For this purpose, we performed a gel mobility shift assay using an upstream promoter DNA fragment (Fig. 3A) and *P. aeruginosa* cell extracts expressing HudR proteins and His-tagged rHudR protein purified from soluble fractions of *E. coli* cells as described in Materials and Methods. The rHudR protein was able to bind to the labeled DNA fragment (Fig. 5A, lane 6). The rHudR-DNA interaction is most likely specific, as assessed by competition assay (Fig. 5B). Whereas we could not observe the binding complex from the PA14 cell extracts, a binding complex was observed for PA14 cells ectopically expressing HudR proteins (Fig. 5A, lanes 4 and 5). Both cell extracts contained different amounts of HudR proteins, as measured by Western blotting (Fig. 5C). More HudR proteins and binding complexes were observed for the cells containing pUCP-HudR than for the cells containing pUCP-HudAR. Since pUCP-HudR has no intrinsic promoter element for the *hudR* gene, the expression of HudR was most likely driven from the *lac* promoter of pUCP18. Whereas pUCP-HudAR has the *hudAp* promoter together with the *lac* promoter in tandem, HudR expression from pUCP-HudAR was in part repressed by HudR itself, which might be attributed to the different amounts of HudR proteins in the two cell extracts.

HudA is a virulence-attenuating factor which is necessary and sufficient for the reduced virulence of the *hudR* mutant. To test for the possibility that the overexpression of HudA by the *hudR* mutant is associated with reduced virulence, we created HudA-expressing constructs (pUCP-HudA and pUCP-HudAR), introduced them into PA14 cells, and then measured growth curves and virulence potentials in *D. melanogaster* and mouse infection experiments. There was no difference in growth characteristics between the wild type and the HudA-expressing bacteria (data not shown). As shown in Fig. 6A and B, HudA overexpression is sufficient for virulence attenuation, regardless of the presence of HudR. PA14 cells containing pUCP-HudA were even less virulent than the *hudR* mutant and PA14 cells containing pUCP-HudAR, most likely due to the higher level of HudA expression. These results demon-

TABLE 3. Transcriptome analysis of the *hudR* deletion mutant

Gene ID[a]	Gene name[b]	Fold upregulation[c]	Description[b]
Genes upregulated in *hudR*			
PA0254	*hudA*	4.3	Homologous to UbiD carboxylase
PA0475		4.3	Probable transcriptional regulator
PA2152		2.8	Probable trehalose synthase
PA2935		2.8	Hypothetical protein
PA3039		2.7	Probable transporter
PA0190		2.6	Probable acid phosphatase
PA2376		2.5	Probable transcriptional regulator
PA2534		2.5	Probable transcriptional regulator
PA0798	*pmtA*	2.5	Phospholipid methyltransferase
PA2517	*xylY*	2.4	Toluate 1,2-dioxygenase beta subunit
PA4127	*hpcG*	2.4	2-Oxo-hept-3-ene-1,7-dioate hydratase
PA0816		2.3	Probable transcriptional regulator
PA1942		2.2	Hypothetical protein
PA4155		2.2	Hypothetical protein
PA4349		2.2	Hypothetical protein
PA1145		2.2	Probable transcriptional regulator
Genes downregulated in *hudR*			
PA0253	*hudR*[d]	−17.4	MarR/SlyA transcriptional regulator
PA1032		−3.0	Probable penicillin amidase
PA1828		−2.9	Probable short-chain dehydrogenase
PA3231		−2.7	Hypothetical protein
PA4990		−2.5	Multidrug efflux transporter
PA2318		−2.5	Hypothetical protein
PA3730		−2.4	Hypothetical protein
PA2669		−2.2	Hypothetical protein
PA2054	*cynR*	−2.1	Transcriptional regulator
PA1090		−2.1	Hypothetical protein
PA2664	*fhp*	−2.1	Flavohemoprotein
PA2449		−2.1	Probable transcriptional regulator
PA0929		−2.0	Two-component response regulator
PA0015		−2.0	Hypothetical protein

[a] Gene ID is the PA number on the PAO1 genome.
[b] Gene names and descriptions are taken from the current annotation information available at http://v2.pseudomonas.com.
[c] The average gene expression level in the *hudR* mutant relative to that in PA14, which was determined by microarray analysis using Affymetrix GeneChips. Shown are genes that were upregulated (positive values) or downregulated (negative values) at a level of statistical significance ($P < 0.05$) as determined by the Mann-Whitney U test.
[d] The decrease of *hudR* in the mutant is most likely attributed to the *hudR* deletion.

strate that the ectopic expression of HudA is sufficient for virulence attenuation in the *hudR* mutant.

To investigate whether or not the *hudR* mutation-induced virulence attenuation requires the functional *hudA* gene, we created *hudA* and *hudAR* deletion mutants as described in Materials and Methods. The *hudA* mutant was no less virulent than PA14 in both *D. melanogaster* and mouse infections (Fig. 6C and D). It was noticeable that the *hudAR* double mutant displayed a wild-type-level virulence potential, indicating that *hudA* is genetically epistatic to *hudR*. Taking these results together, we concluded that the overexpression of HudA is necessary and sufficient for the virulence attenuation of the *hudR* mutant.

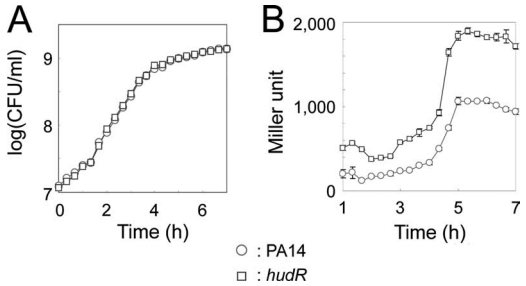

FIG. 4. HudR regulation of *hudAR* transcription. (A) The pQF50-derived *hudARp-lacZ* reporter fusion was introduced into the *hudR* mutant (*hudR*) and PA14 bacteria, and the growth curves were determined based on the viable count, following growth in LB broth. (B) The levels of β-galactosidase at each time point were determined as described in Materials and Methods. Results are the averages from triplicate experiments. The error bars in panel B indicate standard deviations.

DISCUSSION

The results presented in this study demonstrate an exploitation of an invertebrate model host for virulence gene identification of bacterial pathogens, in the sense that we have identified a series of new, as well as already known, virulence factors from this screening. The virulence factors we have identified here represent diverse functional categories and, more importantly, most of them (13 mutants out of 15) are implicated by a mouse infection model as well. Given the nature of the transposon used to generate the mutants (30), the observed that virulence attenuation could be linked to the disruption of a single gene or, in the case of an insertion within an operon, could be due to a polar effect that either increases

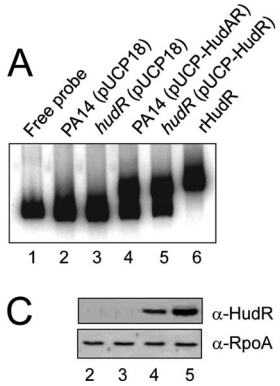

FIG. 5. HudR binds to the upstream region of the *hudAR* operon. (A) Binding of the HudR protein and the *hudAR* promoter. Gel mobility shift assay using the labeled 466-bp *hudAR* promoter fragment and either 20 μg of *P. aeruginosa* cell extracts including HudR proteins as indicated (lanes 2 to 5) or 10 pmol of the purified rHudR protein (lane 6). (B) Specific binding of purified HudR to the *hudAR* promoter. The purified rHudR protein (10 pmol) was incubated with either specific competitors (unlabeled probe DNA) (3 and 30 molar excess for lanes 9 and 10, respectively) or nonspecific competitors (HaeIII digests of pIJ2925) (30 and 300 molar excess for lanes 11 and 12, respectively), prior to the addition of the labeled probe. (C) The amount of the HudR protein in the *P. aeruginosa* bacteria from panel A was assessed by Western blot analysis with antibody against HudR (α-HudR) as described in Materials and Methods. Anti-RpoA antibody (α-RpoA) was used to control the loading amounts.

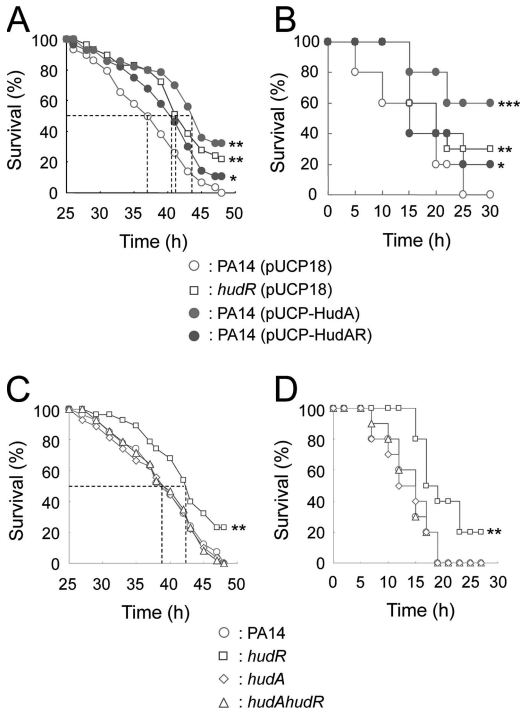

FIG. 6. HudA is necessary and sufficient for the HudR mutant phenotype. (A and B) HudA is sufficient for the *hudR* phenotype. The virulence of the wild-type (PA14) bacteria containing pUCP18-based HudA expression constructs (pUCP-HudA or pUCP-HudAR) was measured in *D. melanogaster* (A) and mouse (B) infection experiments. For mouse infection, 2×10^6 bacterial CFU were used. Survival was determined as described in Materials and Methods. The values are the averages from five replicate experiments for *D. melanogaster* (A) and three experiments for mouse (B) infections. The dotted lines in panel A represent the times required to reach 50% mortality. PA14(pUCP18), PA14 containing the pUCP18 plasmid; *hudR* (pUCP18), the *hudR* deletion mutant containing pUCP18 plasmid; PA14(pUCP-HudA), PA14 containing pUCP18-based expression constructs for HudA; PA14(pUCP-HudAR), PA14 containing pUCP18-based expression constructs for HudA and HudR. The statistical significance for the virulence difference compared with the wild type is indicated (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). (C and D) The *hudA* gene is required for the *hudR* phenotype. The virulence of the *hudA hudR* (*hudAR*) double mutant was measured in *D. melanogaster* (C) and mouse (D) infections. For mouse infection, 2×10^6 bacterial CFU were used. Survival was determined as described in Materials and Methods. The values are the averages of five replicate experiments for *D. melanogaster* (C) and three experiments for mouse (D) infections. The dotted lines in panel C represent the times required to reach 50% mortality. *hudR*, the *hudR* deletion mutant; *hudA*, the *hudA* deletion mutant; *hudAhudR*, the *hudAR* double mutant. The statistical significance for the virulence difference compared with the wild type is indicated (**, $P < 0.01$).

or decreases the expression of the downstream genes. Based on the allelic exchange and Southern blot results, we were able to rule out the secondary mutations that are not linked to the singly inserted transposon. Taking into account that the relatively high proportion of the primary candidates were verified as positive by the secondary screening (43 out of 48 clones), we think that the experimental condition of primary screening might be well optimized for high-throughput identification of *P. aeruginosa* virulence genes by use of *D. melanogaster*.

Previous screens using plant leaf infiltration and *Caenorhabditis elegans* enabled the identification of 21 nonredundant genes necessary for full virulence from a total of 8,000 Tn*phoA* clones of *P. aeruginosa* strain PA14 (29, 34, 37). Out of them, 17 mutants were also shown to be attenuated in a mouse burn model of acute infection. These genes include those involved in quorum sensing (*lasR* and *mvfR*), two-component signal transduction (*gacA*), disulfide bond formation in the periplasm (*dsbA*), and phenazine synthesis (*phzB*), as well as a gene (specific to 33C7) within a pathogenicity island which has been recently characterized (14). Furthermore, in a previous *D. melanogaster*-based screening using 1,500 IS*phoA*/hah transposon clones of *P. aeruginosa* strain PAO1, eight mutants strongly impaired in fly killing, all of which are defective in twitching motility, were identified (7). In the present screen, we have isolated 15 genes from 4,000 Tn*phoA* clones that include already known or presumable virulence genes associated with motilities (*dsbA*, *pilF*, *wspF*, and *flhB*) and iron metabolism (*pvdI*). The *gacA* mutant, which had been independently selected from both plant and *C. elegans* slow-killing screens (34, 37), was not identified in our screen. This is most likely due to the insufficient clone complexity, given that the *gacA* mutant displays virulence attenuation in *D. melanogaster* (22). The virulences of those five mutants were highly attenuated compared to those of the other mutants. This indicates the importance of the pivotal functions that are associated with them (i.e., motilities and iron metabolisms) in virulence mechanisms of *P. aeruginosa*.

Ten virulence-attenuated mutants were newly identified in this screen, which is one of the unique aspects of this study. One mutation (specific to 162C3) is mapped between the coding regions (PA1928 and PA1919) and is not implicated in mouse infection. The other nine genes are supposed to encode

proteins of the following inferred functions: a potential metabolic enzyme (FabF1), three putative membrane proteins (PA0369, PA2002, and PA2113), three putative transcriptional regulators (PA14_36000, PA0272, and HudR), a transposase protein (PA14_35740), and a hypothetical protein (PA2077). Although the precise biochemical functions of these genes as well as their physiological roles remain to be uncovered, it is noteworthy that two genes (PA14_35740 and PA14_36000) are not present in PAO1 but, more interestingly, are located within a potential genetic island that corresponds to the 13th variable region of the PAO1 genome (38). This region most likely represents a pathogenicity island of PA14, which is involved in multihost virulence. This island appears to contain 30 proposed open reading frames (ORFs) (from PA14_35700 to PA14_36030). The 13th variable region contains only 11 coding regions (from PA2218 to PA2228), where the first three genes (PA2218, PA2219, and PA2220) are distinct in terms of the G+C content and distribution among the various strains (38). The average G+C content of the eight ORFs is lower (50.5%) than that of the average G+C content of the PAO1 strain (66.6%), and these ORFs are absent from some strains, indicating that this region may be a horizontally transferred putative alien (12). We are currently characterizing PA14_36000, a gene that encodes a transcription factor similar to PrpR, the propionate catabolism operon regulator in *Salmonella enterica* serovar Typhimurium (18), which contains various domains of functional importance, such as helix-turn-helix (PFAM02954), PAS (PFAM00989), and AAA-type ATPase (PFAM00158) domains. As well, the genetic structures of this island and their potential functions are under intensive investigation with regard to evaluation of the genome and the multihost pathogenesis of *P. aeruginosa*.

We still wonder why the previous *D. melanogaster* screen by D'Argenio et al. (7) enriched the mutants in twitching motility, whereas various functional-category mutants that are also relevant in mouse pathogenesis have been identified in the present screen. One possibility is the strain difference (i.e., PAO1 versus PA14). Another possibility is the clone numbers for both studies, which were insufficient to cover the whole complexity of more than 5,000 coding regions of *P. aeruginosa*. The third possibility, which is most likely, is the difference in infection conditions. In the previous experiment, D'Argenio et al. introduced about 10^3 bacteria, and the time required to reach 100% mortality for the wild-type PAO1 was less than 30 h, which is in clear contrast to our experimental condition. We introduced less than 2×10^2 bacteria, and it takes more than 48 h to reach 100% mortality for both PA14 and PAO1, although PAO1 kills flies slightly faster than does PA14 (data not shown). This explanation is corroborated by the observation that the mortality curves for several PA14 mutants, such as the *gacA* and *flgK* mutants, did not differ from that for PA14 when 10^3 bacteria were introduced (data not shown). Therefore, the infection condition, including the amount of introduced bacteria, is most likely responsible for the difference and thus is critical for the utility and the relevance of the *D. melanogaster* infection setting for the screening procedure.

Two different methods have been employed to infect flies with *P. aeruginosa* bacteria: one is natural infection by providing food containing bacterial cells, and the other is artificial infection by pricking the fly thorax with a needle maculated with bacterial cells. The natural infection usually takes longer (1 or 2 weeks) to kill flies than the artificial infection (1 or 2 days), and the mortality by natural infection for the wild-type *P. aeruginosa* is usually less than 50% (reference 6 and data not shown). More importantly, in case of *P. entomophila*, a recently isolated natural pathogen of *D. melanogaster*, natural infection (i.e., oral administration) could trigger both local (i.e., gut) and systemic immune responses, but the local immune responses contribute preponderantly to resistance against *P. entomophila* infection (27). Based on the fact that artificial infection by pricking triggers the systemic immune responses predominantly, the molecular interactions between *P. aeruginosa* virulence factors and *D. melanogaster* immunity components differ between the two infection methods. Nevertheless, *P. aeruginosa* is not a natural pathogen toward *D. melanogaster*, which may have some neuronal responses to sense the presence of harmful bacteria in the food, like *C. elegans* does (39). Given these considerations, we are unable to control the amount of bacterial cells that entered the fly body. Therefore, artificial infection by pricking should be exploited to infect flies with a large number of *P. aeruginosa* clones for the screening procedure. Our present approach to infect with less than 2×10^2 bacteria by pricking appears to be successful in terms of high-throughput screening for virulence-attenuated mutants. Currently, nonredundant saturated transposon library sets for both PAO1 and PA14 strains are available (21, 26), and the use of invertebrate model hosts and the libraries combined with the appropriate experimental conditions to manifest the pathogenic interaction will enable the genome-wide identification of the shared sets of virulence factors, which will significantly contribute to the elucidation of the molecular pathogenesis mechanisms that underlie acute infection by *P. aeruginosa* strains.

The newly identified HudR repressor and its target, HudA, the ectopic expression of which attenuates the virulence of *P. aeruginosa*, are noticeable in view of two aspects: first, the deletion of both genes (i.e., *hudAR* deletion) is dispensable for growth as well as survival in various stressful conditions that include treatments with oxidants, reductants, salts, and heat (data not shown); second, *hudAR* gene expression was markedly increased during the stationary growth phase. We observed that the stationary-phase induction of *hudAR* gene expression was still observed in the *rpoS*, *rpoN*, *lasR*, *mvfR*, and *rhlR* mutants as well as in the *rpoS lasR rhlR* triple mutant (data not shown). Since the proliferation of *P. aeruginosa* might be restricted by unfavorable conditions that the host environments usually provide, the genes that are expressed during the stationary growth phase are simply supposed to support the virulence mechanisms. Since HudR represses the expression of HudA and HudR, constituting an autoregulation, the steady-state level of HudA and HudR proteins and the signal to induce *hudAR* expression during the stationary growth phase should be addressed to uncover the physiological role of this operon.

Because HudA is a potential membrane protein like UbiD carboxylase, we hypothesize that some pivotal membrane functions might be disturbed by HudA ectopic expression. We are currently determining the localization of HudA as well as the membrane properties and dynamics, based on the observation that the membrane incorporation of a lipophilic fluorescent

probe (1,6-diphenyl-1,3,5-hexatriene) was markedly lower in the *hudR* mutant than in the wild-type bacteria (data not shown). Although HudA is highly homologous to UbiD carboxylase, its function as well as its physiological role may be distinguished from those of the latter. Using transmission electron microscopy, we could not find any alteration in the flagellation of the cells overexpressing HudA, although there is a report that the induction of a functional ubiquinone biosynthesis pathway affected flagellation in *Salmonella enterica* serovar Typhimurium (20). We did not see any virulence alteration by overexpression of UbiD either (data not shown). Furthermore, the *hudA*-like genes are found exclusively in *P. aeruginosa* strains and in some ascomycete fungi. This relatively confined gene distribution, together with the understanding of the conserved residues within HudA and HudA-like proteins and the physiology of the fungal species possessing HudA-like proteins, may help to elucidate the physiological and biochemical functions of HudA in *P. aeruginosa* pathogenesis.

As a multihost pathogen, *P. aeruginosa* is able to infect various model hosts, with a broad spectrum of pathologies depending on the infection conditions as well as the host models exploited. It is highly versatile in its adaptation to the susceptible host tissues, not only as an initial colonizer resulting in acute infection but also as a persistent survivor as a result of chronic infection. Some subsets of virulence factors are independently and separately implicated in the different pathological consequences that this pathogen may cause; for example, motility and toxin production are likely associated with acute infections, whereas chronic infections are likely implicated in biofilm formation. The recent study by Smith et al. (36) suggests that the genetic adaptation of *P. aeruginosa* during chronic infections favors the loss of virulence functions for acute infections. To the best of our knowledge, there are no proper invertebrate model hosts relevant to the study of chronic infections. In this regard, the current exploitations of invertebrate model hosts, including plants, nematodes, and insects, may have definite limitations, although the experimental settings should be improved. Therefore, we need to establish more and more-relevant model hosts based on a better understanding of the characteristics of the established model hosts in terms of the pathological consequences and molecular interactions caused by *P. aeruginosa* infections.

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