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# Identification of Virulence Genes in a Pathogenic Strain of *Pseudomonas aeruginosa* by Representational Difference Analysis

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***Pseudomonas aeruginosa*** **is an opportunistic pathogen that may cause severe infections in humans and other vertebrates. In addition, a human clinical isolate of *P. aeruginosa*, strain PA14, also causes disease in a variety of nonvertebrate hosts, including plants, *Caenorhabditis elegans*, and the greater wax moth, *Galleria mellonella*. This has led to the development of a multihost pathogenesis system in which plants, nematodes, and insects have been used as adjuncts to animal models for the identification of *P. aeruginosa* virulence factors. Another approach to identifying virulence genes in bacteria is to take advantage of the natural differences in pathogenicity between isolates of the same species and to use a subtractive hybridization technique to recover relevant genomic differences. The sequenced strain of *P. aeruginosa*, strain PAO1, has substantial differences in virulence from strain PA14 in several of the multihost models of pathogenicity, and we have utilized the technique of representational difference analysis (RDA) to directly identify genomic differences between *P. aeruginosa* strains PA14 and PAO1. We have found that the *pilC*, *pilA*, and *uvrD* genes in strain PA14 differ substantially from their counterparts in strain PAO1. In addition, we have recovered a gene homologous to the *ybtQ* gene from *Yersinia*, which is specifically present in strain PA14 but absent in strain PAO1. Mutation of the *ybtQ* homolog in *P. aeruginosa* strain PA14 significantly attenuates the virulence of this strain in both *G. mellonella* and a burned mouse model of sepsis to levels comparable to those seen with PAO1. This suggests that the increased virulence of *P. aeruginosa* strain PA14 compared to PAO1 may relate to specific genomic differences identifiable by RDA.**

*Pseudomonas aeruginosa* is an opportunistic pathogen that frequently causes severe systemic infections, particularly in patients with cystic fibrosis, burns, and immunosuppression (2, 3, 48, 52). The versatility of *P. aeruginosa* may be a consequence of its ability to produce a wide variety of both cell-associated and extracellular virulence factors. Cell-associated virulence factors include pili, flagellae, lipopolysaccharide, a type III secretion system, and alginate. Secreted products include low-molecular-weight toxins, such as phenazines, rhamnolipid, and cyanide, and numerous protein virulence factors, including ADP-ribosylating enyzmes, proteases, and phospholipases (12, 16, 42, 62). Additional virulence factors include proteins required for the expression or the secretion of these molecules, often in response to particular environmental stimuli (17, 18).

A soil inhabitant, *P. aeruginosa* is widely distributed in the natural environment and can also act as a plant pathogen. Recently, Rahme et al. (45) have exploited the broad host range of this pathogen and have shown that a clinical isolate of *P. aeruginosa*, strain PA14, is capable of causing disease both in an *Arabidopsis thaliana* leaf infiltration model and in a mouse full-thickness skin thermal burn model. Furthermore, mutations in a variety of PA14 genes reduced the virulence of this strain for both plants and mice, suggesting that at least some of the mechanisms of pathogenesis of *P. aeruginosa* infection may be conserved in evolutionarily divergent hosts (44).

These results have subsequently been extended to show that *P. aeruginosa* can also act as a pathogen for a variety of additional nonvertebrate hosts, including *Caenorhabditis elegans* (33, 58, 59), *Drosophila melanogaster* (13; Mahajan-Miklos et al., unpublished data), and the greater wax moth, *Galleria mellonella* (25). This has led to the development of a multihost pathogenesis system in which plants, nematodes, and insects have been used as adjuncts to animal models for the identification and study of bacterial virulence factors of *P. aeruginosa*. Indeed, random transposon mutagenesis has been used to create 8,000 individual mutants of strain PA14, and these clones have been screened for reduced virulence compared to the wild-type parent by using a plant leaf infection model (46), in fast- and slow-killing assays in *C. elegans* (33, 58, 59) and in the *G. mellonella* model system (25). The relevance to mammalian pathogenesis of virulence factors identified using these screens has been confirmed by using a mouse full-thickness burn model (54). Remarkably, of 20 genes in *P. aeruginosa* strain PA14 that are required for pathogenesis in at least one of the three different invertebrate hosts (a plant, a nematode, or an insect), 17 were also required for full pathogenicity in a mouse burn model. Of these 17 genes, eight encode novel proteins and three encode proteins not previously known to be involved in

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bacterial pathogenesis. Many classes of genes were identified, including genes encoding proteins involved in transcriptional and posttranscriptional regulation, efflux systems, biosynthetic enzymes involved in phenazine production, and proteins of unknown function (32). Since the 8,000 mutants screened so far represent approximately 25 to 33% of the total number that need to be screened to ensure a 95% probability of testing a mutation in each *P. aeruginosa* gene in these assays, many additional factors involved in the pathogenicity of *P. aeruginosa* may remain to be discovered.

Another approach to identifying virulence factors in bacteria is to take advantage of naturally occurring differences in pathogenicity between isolates of the same species, utilizing one of a variety of subtractive techniques to recover genes present in one isolate but not the other, such as those found on pathogenicity islands. One such technique is representational difference analysis (RDA), a procedure involving subtractive hybridization and kinetic enrichment that has been used previously to recover differences between two complex genomes, including identifying the genome of human herpesvirus 8 in the tissue of patients with Kaposi's sarcoma (29, 30). RDA has also been modified to utilize cDNA as the starting material, thereby allowing analysis of differential gene expression (24). Recently, RDA was adapted for use in detecting and cloning genomic differences between two closely related bacterial species or isolates of the same species (8, 41, 60).

In this study, we have taken advantage of the fact that the sequenced strain of *P. aeruginosa*, strain PAO1, has substantial differences in virulence from strain PA14 in several of the multihost models of pathogenicity described above. Strain PAO1 has been used in numerous previous investigations related to the genetics and virulence of *P. aeruginosa* (21, 36, 37, 38). We utilized the technique of RDA to directly detect genomic differences between *P. aeruginosa* strains PA14 and PAO1 and examined whether any of the identified genomic differences relate to the differences in virulence between the two strains in the various models of pathogenesis. We found that the *pilC*, *pilA*, and *uvrD* genes in *P. aeruginosa* strain PA14 differ substantially from their counterparts in strain PAO1. We also recovered a *ybtQ* gene homolog that is specifically present in strain PA14 but absent in strain PAO1. We demonstrated that mutation of the *ybtQ* homolog in *P. aeruginosa* strain PA14 significantly attenuates the virulence of this strain in both *G. mellonella* and a burned mouse model of sepsis, to levels comparable to those seen with PAO1, suggesting that the increased virulence of *P. aeruginosa* strain PA14 compared to PAO1 may relate to specific genomic differences identifiable by RDA.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains and plasmids used in this study are shown in Table 1. *P. aeruginosa* strain PA14 is a human clinical isolate used for identification of novel virulence-related genes (45). *P. aeruginosa* strain PAO1 has been studied extensively in many laboratories (21, 36, 37, 38), and the genomic sequence has been determined (55). All strains were maintained at −70°C in Luria-Bertani (LB) medium containing 15% glycerol. LB broth and agar were used for the growth of *P. aeruginosa* and *Escherichia coli* strains at 37°C. Chelex-100 treated syncase medium (15) was used for the low-iron growth conditions for the *P. aeruginosa ybtQ* mutant. Antibiotic concentrations were as follows: for *E. coli*, ampicillin at 100 μg $ml^{-1}$; for *P. aeruginosa*, rifampin at 100 μg $ml^{-1}$ and carbenicillin at 300 μg $ml^{-1}$.

**Molecular genetic techniques.** Isolation of plasmid DNA, restriction enzyme digests, and agarose gel electrophoresis were performed according to standard molecular biological techniques (50). Restriction enzyme-digested chromosomal and plasmid DNA fragments were separated on 0.8% agarose gels; fragments of interest were cut from the gel under UV illumination and purified with a QIAEX II gel extraction kit (Qiagen Inc., Valencia, Calif.).

DNA sequencing was performed at the Massachusetts General Hospital Department of Molecular Biology in the DNA Sequencing Core Facility using ABI Prism DiTerminator cycle sequencing with AmpliTaq DNA polymerase FS and an ABI377 DNA sequencer (Perkin-Elmer/Applied Biosystems, Foster City, Calif.). The sequences obtained were analyzed against the *P. aeruginosa* PAO1 genome sequence generated by the *P. aeruginosa* genome project (Cystic Fibrosis Foundation and Pathogenesis Corporation) and at the National Center for Biotechnology Information via the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

**Representational difference analysis.** The procedure of RDA was originally described by Lisitsyn et al. (29) and adapted to the comparison of bacterial strains by Tinsley and Nassif (60) and Calia et al. (8). In the present study, 2 μg of DNA from *P. aeruginosa* strain PA14 was cleaved with *Sau*3AI, precipitated with ethanol-sodium acetate, and ligated for 18 h at 16°C with 5 nmol of the oligonucleotide adapter pair (RSau24 [5′-AGCACTCTCCAGCCTCTCACCG CA-3′] and RSau12 [5′-GATCTGCGGTGA-3′]). The mixture was gel purified on 2% low-melting-point agarose (taking fragments above 200 bp) to remove unincorporated primers, phenol purified, precipitated, and redissolved in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) at a DNA concentration of 0.1 μg $\mu l^{-1}$. This procedure results in DNA fragments whose two 5′ ends are covalently linked to the 24-base adapter (RSau24). To prepare the subtracting DNA, chromosomal DNA of *P. aeruginosa* strain PAO1 was sheared by repeated passage through a 30-gauge hypodermic needle to give fragments ranging from about 3 to 10 kbp. The DNA was repurified by phenol extraction, precipitated, and redissolved in TE buffer at a concentration of 0.1 μg $\mu l^{-1}$. The first subtractive hybridization was performed with 40 μg of *P. aeruginosa* strain PAO1 subtracting DNA and 400 ng of *Sau*3AI-digested, RSau adapter-linked PA14 DNA fragments. The DNA was mixed, ethanol precipitated, and redissolved in 8 μl of EE buffer (10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[3-propanesulfonic acid], 1 mM EDTA [pH 8.0]). The liquid was overlaid with 30 μl of mineral oil, denatured at 100°C for 2 min, and then placed at 67°C. After the addition of 2 μl of 5 M NaCl to the aqueous phase, the mixture was left to hybridize at 67°C for 20 h. The reaction mixture was then diluted 10-fold with preheated EE buffer–NaCl and immediately placed on ice. A portion of the subtraction mixture (10μl) was diluted into 400 μl of PCR mix [67 mM Tris-HCl (pH 8.9)], 16 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1 mg of bovine serum albumin $ml^{-1}$, a 0.125 mM concentration of each deoxynucleoside triphosphate, 15 U of *Taq* polymerase per ml) to fill in the ends corresponding to the 24-base adapter (RSau24). The reaction mixture was diluted 10-fold further, and PCR amplification was performed on 400 μl of the dilution. After denaturation for 5 min at 94°C and addition of the appropriate 24-base oligonucleotide (RSau24 primer), the mixture was amplified by PCR (10 cycles of 1 min at 95°C and 3 min at 72°C, with the last cycle followed by an extension at 72°C for 10 min). Single-stranded DNA molecules present after amplification were degraded by a 30-min incubation with 20 U of mung bean nuclease, diluted (1:5) in 50 mM Tris-HCl (pH 8.9), and then heated to 95°C for 5 min to inactivate the enzyme. A portion (40 μl) of the solution was further amplified for 20 cycles under the conditions described above. The amplified *P. aeruginosa* DNA fragments were separated by agarose gel electrophoresis from the primers and high-molecular-weight subtracting DNA. The RSau adapters were cleaved from the PCR products by digestion with *Sau*3AI, and 2 nmol of the second-round adapters (JSau24 [5′-ACCGACGTCGACTATCCATGAACA-3′] and JSau12 [5′-GATCTGTTC ATG-3′]) were ligated with 2 μg of the first-round difference products in a volume of 50 μl. The ligated fragments were gel purified, phenol extracted, and ethanol precipitated. A second round of subtractive hybridization and PCR enrichment was performed with 400 ng of first-round products religated to the JSau adapters and 40 μg of sheared DNA from *P. aeruginosa* strain PAO1 as described above. Fragments amplified from the second round were cleaved with *Sau*3AI, gel purified, and cloned into the pEX18 vector (23) digested with *Bam*HI. The recombinant plasmids were maintained in *E. coli* strain DH5α. DNA sequences of the RDA products corresponding to the inserted DNA were determined with primers flanking the polylinker site of pEX18. The pool of fragments obtained after the second round of RDA was also tested by Southern hybridization to ensure that they were absent in PAO1 and present in PA14.

**Southern hybridization.** Bacteria from 10 ml of LB broth were resuspended in 1 ml of 10 mM Tris-HCl (pH 8.0)–10 mM EDTA–100 mM NaCl containing 2 μg of RNase A. After addition of 50μl of 20% sodium dodecyl sulfate and incubation at 65°C for 30 min, the mixture was digested for 2 h at 37°C with proteinase K (100 μg). The solution was then extracted once with an equal volume of phenol

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant genotype and/or phenotype[a] | Source or reference |
|---|---|---|
| *P. aeruginosa* | | |
| PAO1 | Wild-type laboratory strain | Lab collection |
| PA14 | Human clinical isolate; $Rif^r$ | 45 |
| JY2M | *pilC* mutant of PA14; $Rif^r$ $Cb^r$ | This study |
| JY5M | *pilA* mutant of PA14; $Rif^r$ $Cb^r$ | This study |
| JY11M | *ybtQ* mutant of PA14; $Rif^r$ $Cb^r$ | This study |
| JY15M | *uvrD* mutant of PA14; $Rif^r$ $Cb^r$ | This study |
| *E. coli* | | |
| DH5α | $F^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* $\lambda^-$ ϕ80 *dlacZ*Δ*M15* | Gibco BRL |
| SM10 | *thi-I thi leu tonA lacY supE recA*::*RP4-2-Tc*::*Mu*; $Km^r$ | Lab collection |
| Plasmids | | |
| pUC19 | Cloning vector; $Ap^r$ | Lab collection |
| pEX18 | Suicide vector for *P. aeruginosa*; *oriT*$^+$ *sacB*$^+$, gene replacement vector with MCS from pUC18; $Ap^r$ | 23 |
| pGEM-T Easy | PCR cloning vector; $Ap^r$ | Promega |
| pJY1 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY2 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY3 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY4 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY5 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY6 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY8 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY10 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY11 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY12 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY13 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY15 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY16 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY17 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY19 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY20 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY22 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY23 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY24 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJY25 | pEX18 derivative carrying RDA product; $Ap^r$ | This study |
| pJYP25 | pGEM-T Easy with a 3.0-kbp fragment of *pilABC* gene cluster; $Ap^r$ | This study |
| pJY2A | pGEM-T Easy with a 1,160-bp IPCR product of *pilC*; $Ap^r$ | This study |
| pJY5A | pGEM-T Easy with a 1,030-bp IPCR product of *pilA*; $Ap^r$ | This study |
| pJY15A | pGEM-T Easy with a 1,150-bp IPCR product of *uvrD*; $Ap^r$ | This study |
| pJY2-1 | pGEM-T Easy with a 600-bp internal fragment of *pilC*; $Ap^r$ | This study |
| pJY5-1 | pGEM-T Easy with a 500-bp internal fragment of *pilA*; $Ap^r$ | This study |
| pJY11-1 | pGEM-T Easy with a 1.3-kbp internal fragment of *ybtQ*; $Ap^r$ | This study |
| pJY15-1 | pGEM-T Easy with a 1.1-kbp internal fragment of *uvrD*; $Ap^r$ | This study |
| pJY2M | pEX18 derivative carrying a 600-bp internal fragment of *pilC*; $Ap^r$ | This study |
| pJY5M | pEX18 derivative carrying a 500-bp internal fragment of *pilA*; $Ap^r$ | This study |
| pJY11M | pEX18 derivative carrying a 1.3-kbp internal fragment of *ybtQ*; $Ap^r$ | This study |
| pJY15M | pEX18 derivative carrying a 1.1-kb internal fragment of *uvrD*; $Ap^r$ | This study |
| pJYYBT | 2.1-kb *Eco*RI fragment containing the intact *ybtQ* gene cloned into pUC19 | This study |

[a] $Ap^r$, ampicillin resistance; $Cb^r$, carbenicillin resistance; $Km^r$, kanamycin resistance; $Rif^r$, rifampin resistance.

(pH 8.0), twice with phenol-chloroform-isopropanol (25:24:1), and once with chloroform-isopropanol (24:1). The solution was overlaid with an equal volume of ethanol and cooled to 0°C, and the DNA was spooled from the interface by mixing with a glass Pasteur pipette. DNA was washed in 70% ethanol, partially dried, and redissolved in TE buffer. The concentration of DNA was determined by UV spectrophotometry.

After digestion of purified DNA with *Sau*3AI and separation by agarose gel electrophoresis, Southern blotting was performed by capillary transfer onto Hybond-$N^+$ positively charged nylon membranes (Amersham Pharmacia Biotech., Piscataway, N.J.). Hybridization of labeled probes and detection were performed with an enhanced chemiluminescence kit (Amersham) as described by the manufacturer.

**IPCR.** To obtain chromosomal sequences flanking RDA fragments from *pilC*, *pilA*, and *uvrD*, we performed partial inverse PCR (IPCR) (39). *P. aeruginosa* strain PA14 chromosomal DNA (10 μg) was partially digested with *Sau*3AI at 2 U $\mu g^{-1}$ for 1 h. The reactions were stopped by heating at 65°C for 20 min, and an aliquot of the reaction mixture was run on a 0.8% agarose gel to check the extent of cutting. The rest of the DNA was ethanol precipitated and resuspended in 1× ligation buffer to a concentration of 5 ng $\mu l^{-1}$. $T_4$ DNA ligase was added, and chromosomal fragments were allowed to self-ligate at 22°C for 4 h. Ligation was stopped by heating to 65°C for 20 min followed by phenol extraction, ethanol precipitation, and resuspension in TE buffer at a DNA concentration of 100 ng $\mu l^{-1}$. Two primers (5′-CATTTAGGGAAGCTCATCA-3′ and 5′-GAACTGTG GGACCACTTTTATC-3′ [*pilC*], 5′-CTAGTGAAAGGGCAGGCCT-3′ and 5′-GGCATGCAAGATGCTTTA-3′ [*pilA*], 5′-ACTCTTCTTCAAGTTCGGA-3′ and 5′-CAGATGCAGGGCAAGTTCT-3′ [*uvrD*]) facing outwards from the RDA sequences of the *pilC*, *pilA*, and *uvrD* genes were used to carry out each IPCR. The PCR reaction mixture (20 μl) contained 200 ng of religated DNA, 0.2

mM concentrations of each deoxynucleoside triphosphate, 2 mM magnesium, 1 μM concentrations of each primer, and 1 U of *Taq* DNA polymerase in 1× buffer supplied by the manufacturer. PCR was performed for 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The largest IPCR products (1,160 bp, *pilC*; 1,030 bp, *pilA*; and 1,150 bp, *uvrD*) were excised from a gel, purified with the QIAEX II gel extraction kit, and cloned into the PCR cloning vector pGEM-T Easy, to generate pJY2A, pJY5A, and pJY15A. DNA sequencing confirmed plasmids carrying the correct insert corresponding to each RDA product.

To confirm that the *pilC* fragment in pJY2A and the *pilA* fragment in pJY5A were located near each other in the *pilABC* gene cluster, a 3.0-kbp fragment of *pilABC* was generated by PCR using the JY2F primer (5′-TGATGAGCTTCC CTAAATG-3′; 5′ sequence of pJY2) in combination with the JY5G primer (5′-ACTGGACATAGGGGGTAAG-3′; 3′ sequence of pJY5). The amplified product was cloned into the PCR cloning vector pGEM-T Easy and designated pJYP25.

**Cloning of the *ybtQ* gene from a plasmid library.** Chromosomal DNA of *P. aeruginosa* strain PA14 was fully digested with various restriction enzymes, electrophoresed, and transferred to Hybond-$N^+$ positively charged nylon membranes (Amersham). Southern blot analysis was performed as described above, using the insert of plasmid pJY11 as a probe; digestion with *Eco*RI gave a single hybridizing fragment of 2.1 kbp. We digested chromosomal DNA of PA14 with *Eco*RI, recovered 2.0 to 3.0-kbp fragments from the agarose gel after electrophoresis and extracted these with the QIAEX II gel extraction kit. The recovered DNA fragments were ligated into the *Eco*RI site of pUC19, and the resulting plasmids were transformed into ultracompetent *E. coli* DH5α. This library of plasmids was screened by colony blot hybridization (50) using the insert DNA of plasmid pJY11 as the probe. Individual positive plasmid clones with appropriate insert sizes were verified by Southern blot hybridization, and the DNA sequence of the 2,126-bp insert in plasmid pJYYBT was determined. DNA and deduced amino acid sequences were analyzed with CloneMap version 2.11 (CGC Scientific, Inc., Ballwin, Mo.) and the WU-Blast2 program in EMBL (European Bioinformatics Institute) (http://www2.ebi.ac.uk/blast2/). Motif analysis of deduced amino acid sequences was performed with the Expert Protein Analysis System (ExPASy) Molecular Biology Server (http://www.expasy.ch).

**Strain constructions.** *P. aeruginosa* strain PA14 *pilC*, *pilA*, *ybtQ*, and *uvrD* mutants JY2M, JY5M, JY11M, and JY15M were constructed by inserting a suicide vector, containing an internal fragment of each gene, into the chromosome of PA14. A 700-bp internal fragment of *pilC* from PA14 was amplified by PCR (94°C for 3 min, 25 cycles at 94°C for 1 min, 50°C for 2 min, 72°C for 5 min, 72°C for 10 min), using primers JY2R (5′-GCAGCAAGGTCAAAGGAGAG-3′) and JY2L (5′-TGAGCTTCCCTAAATGCAAAAG-3′), and cloned into the PCR cloning vector pGEM-T Easy vector to create plasmid pJY2-1. The 500-bp, 1.3-kbp, and 1.1-kbp internal fragments of *pilA*, *ybtQ*, and *uvrD* genes were similarly amplified with primer pairs JY5R (5′-GAAAGGCTTTACCTTGAT-3′) and JY5L (5′-AGGAGCGAAACGAGCCG-3′), JY11R (5′-CTACGCAAT CATGGCAGTA-3′) and JY11L (5′-CGATTCCATGCAGCCTGTGT-3′), and JY15R (5′-CACGCATGCATTGTAGCGA-3′) and JY15L (5′-GATCGGTAG CGCAAAACT-3′), respectively, and cloned into pGEM-T Easy to generate plasmids pJY5-1, pJY11-1, and pJY15-1. The *Sac*I-*Sph*I fragments from pJY2-1, pJY5-1, pJY11-1, and pJY15-1 were cloned into the *Sac*I and *Sph*I sites in the polylinker of pEX18 to generate plasmids pJY2M, pJY5M, pJY11M, and pJY15M. Plasmid constructions were verified by DNA sequencing.

Plasmids pJY2M, pJY5M, pJY11M, and pJY15M were transformed into *E. coli* SM10 and subsequently transferred to *P. aeruginosa* strain PA14 by conjugation. Carbenicillin- and rifampin-resistant transconjugants contained the mobilized plasmid integrated into the genome by homologous recombination. Insertional mutation was confirmed by Southern hybridization of chromosomal DNA of each mutant strain compared with PA14, using the inserts of plasmids pJY2, pJY5, pJY11, and pJY15 as probes.

**Virulence testing.** We determined the virulence of various strains of *P. aeruginosa* for a number of nonvertebrate hosts, including *G. mellonella* (caterpillars) and *C. elegans*. To examine virulence in *G. mellonella*, overnight cultures were grown in LB broth, diluted 1:100 in the same medium, and grown to an optical density at 600 nm of 0.3 to 0.4. Cultures were pelleted and resuspended in 10 mM $MgSO_4$. After dilution to an optical density at 600 nm of 0.1 with 10 mM $MgSO_4$, serial 10-fold dilutions were made in 10 mM $MgSO_4$ with 2 mg of rifampin per ml for *P. aeruginosa* strain PA14 (and derivatives) and 0.5 μg of ampicillin per ml for strain PAO1. A 10-μl Hamilton syringe was used to inject 5-μl aliquots into individual fifth-instar *G. mellonella* larvae (Van der Horst Wholesale, St. Marys, Ohio), via the hindmost left proleg. A series of 10-fold serial dilutions containing from $10^6$ to 0 bacteria were injected into the *G. mellonella* larvae. Ten larvae were injected at each dilution, and larvae were scored as live or dead after 60 h at 25°C. Data from three independent experiments were combined. The Systat computer program was used to fit a curve to the infection data using the following formula: $Y = A + (1 - A)/\{1 + \exp[B - G \cdot \ln(X)]\}$, where $X$ is the number of bacteria injected, $Y$ is the fraction of larvae killed by the infection, $A$ is the fraction of larvae killed by control injections, and $B$ and $G$ are parameters which are varied for optimal fit of the curve to the data points. The 50% lethal dose ($LD_{50}$) is calculated from the curve. Statistical significance of differences was determined by using Fisher's exact test. Slow killing assays in *C. elegans* were performed as described previously (58, 59).

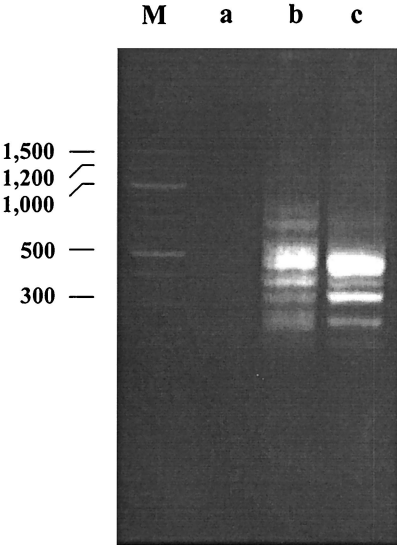


FIG. 1. Agarose gel electrophoresis (2% gel) of RDA products between *P. aeruginosa* PA14 and PAO1. The tester DNA amplicons before DNA hybridization and amplification (a) and the difference products after the first (b) and second (c) DNA hybridization-PCR amplification steps are shown. M, Molecular size markers; sizes are on the left, in base pairs.

To examine virulence of various strains in mice, a 5% total surface area burn was fashioned on the outstretched abdominal skin of 6-week-old male AKR/J mice weighing between 25 and 30 g (The Jackson Laboratories, Bar Harbor, Maine). Immediately following the burn, mice were injected subcutaneously with $5 \times 10^5$ CFU of the *P. aeruginosa* strain being analyzed, and the number of animals that died as a result of sepsis was monitored each day for 10 days. For each strain, data from two independent experiments (eight mice per experiment) were combined (except that *P. aeruginosa* strain PAO1 was tested only once). Animal study protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

**Nucleotide sequence accession numbers.** The sequences of the *P. aeruginosa* PA14 *ybtQ*, *uvrD*, *pilC*, and *pilA* genes have been submitted to the GenBank database; accession numbers are AY049068, AY049069, AY049070, and AY049071.

## RESULTS

**Construction of a library of fragments of *P. aeruginosa* PA14 DNA not found in the genome of PAO1.** Using RDA, we subtracted total genomic DNA of the sequenced strain, *P. aeruginosa* PAO1, from strain PA14. Following two rounds of subtractive and kinetic enrichment, agarose gel electrophoresis of the amplified difference products revealed a small number of DNA fragments between 100 and 800 bp, with bands in the 200- to 500-bp range present in the greatest abundance (Fig. 1). We confirmed that the sequences amplified by RDA were specific to *P. aeruginosa* PA14 by using the pool of second-round difference products to probe genomic DNA of *P. aeruginosa* strains PAO1 and PA14 (Fig. 2).

We ligated the pool of second-round difference products

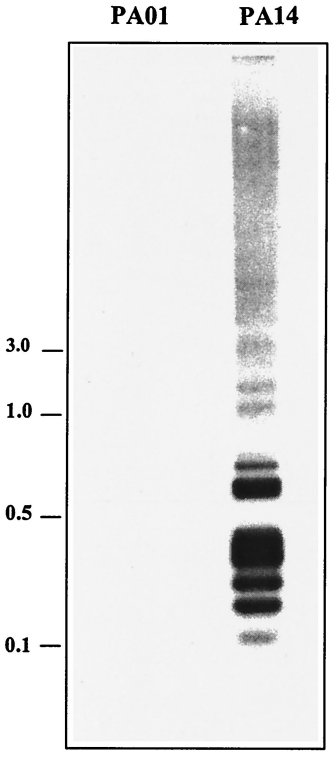


FIG. 2. Southern blot analysis confirms that the second-round RDA products are unique to PA14. Chromosomal DNA was isolated from *P. aeruginosa* strains PAO1 and PA14, digested with *Sau*3AI, separated on a 0.8% agarose gel, transferred to a membrane, and hybridized with a labeled pool of the second-round products. Molecular sizes are on the left, in kilobase pairs.

into pEX18 and recovered a total of 57 clones. Of these, the insert sequences of 20 representative clones, designated pJY, were determined using primers flanking the polylinker site of pEX18 (Table 2). When the sequences of these 20 inserts were compared to the *P. aeruginosa* PAO1 genome sequence (http://www.pseudomonas.com/GenomeSearch.asp), none showed a match, as expected from the Southern analysis results. Of the 20 clones chosen for further analysis, 14 showed homology to known genes, while 5 had no significant matches by BLAST search (http://www.ncbi.nlm.nih.goy/blast) (Table 2). One clone, pJY25, was a sibling of another (pJY13).

We focused our attention on the inserts in pJY2, pJY5, pJY11, and pJY15, which BLAST searches revealed to be homologs, respectively, of the type IV fimbrial assembly protein, PilC, in *P. aeruginosa*, the type IV pilin, PilA, in *P. aeruginosa*, the ABC transporter protein, YbtQ, in *Yersinia pestis*, and the DNA helicase, UvrD, in *Chlamydia trachomatis*.

**Cloning and sequencing of the *pilA*, *pilC*, and *uvrD* genes from *P. aeruginosa* strain PA14.** We recovered 1,160-, 1,030-, and 1,150-bp fragments containing *P. aeruginosa* strain PA14 *pilC*, *pilA*, and *uvrD* genes by inverse PCR and cloned these into pGEM-T Easy to construct plasmids pJY2A, pJY5A, and pJY15A, respectively.

The deduced amino acid sequence encoded by bp 1 to 1,160 in the insert of pJY2A showed 72% identity and 79% similarity by BLAST search to the type IV fimbrial assembly protein PilC in *P. aeruginosa* strain PAO1. A pairwise BLAST alignment (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) of the PilC amino acid sequences from *P. aeruginosa* strains PAO1 and PA14 showed that these genes shared a number of variable regions separated by conserved domains; the RDA product originally isolated in pJY2 was derived from one of the variable regions that differ substantially between PA14 and PAO1.

A BLAST search using the insert in pJY5A showed 91% identity over 179 amino acids to the type IV pilin from the *P. aeruginosa* G7 and G9 strains (53). The N-terminal 30 amino acids showed significant homology to various other members of the type IV group A prepilins, including PilA of *P. aeruginosa* strain PAO1 (26), FimA of *Dichelobacter nodosus* (6), and PilE of *Neisseria gonorrhoeae* (5). The C-terminal regions of these proteins were more variable, and the RDA product originally

TABLE 2. Homologies of the *P. aeruginosa* PA14 strain-specific RDA clones

| Clone | Length (bp) | Homology with BLASTX | GenBank accession no. |
|---|---|---|---|
| pJY1 | 195 | Ubiquitin-specific protease Ubp7p of *Saccharomyces cerevisiae* | NC001141 |
| pJY2 | 175 | PilC of *P. aeruginosa* | M32066 |
| pJY3 | 138 | Chlorophyll *b* synthetase of *Dunaliella salina* | AB021312 |
| pJY4 | 338 | KIAA0054 gene product; helicase of *Homo sapiens* | XP008261 |
| pJY5 | 348 | Type 4 pilin of *P. aeruginosa* | L37109 |
| pJY6 | 180 | Unknown | |
| pJY8 | 397 | Hypothetical protein Y68A4A.10 of *C. elegans* | AL021503 |
| pJY10 | 117 | Unknown | |
| pJY11 | 196 | YbtQ, ABC transporter, ATP-binding component of *Y. pestis* | AF091251 |
| pJY12 | 234 | Unknown | |
| pJY13 | 215 | Alpha-1 tubulin of *C. elegans* | D16439 |
| pJY15 | 368 | UvrD, DNA helicase of *C. trachomatis* | AE001331 |
| pJY16 | 116 | Unknown | |
| pJY17 | 230 | Patched-related proteins of *C. elegans* | AC006670 |
| pJY19 | 274 | Unknown protein of *Pasteurella multocida* | AE006141 |
| pJY20 | 128 | PilC of *N. gonorrhoeae* | AJ00121 |
| pJY22 | 217 | Unknown | |
| pJY23 | 264 | Nuclear receptor NHR-18 of *C. elegans* | AF083232 |
| pJY24 | 366 | Hypothetical 119.5K protein of *Micrococcus luteus* | JQ0405 |
| pJY25 | 215 | Alpha-1 tubulin of *C. elegans* | D16439 |

isolated in pJY5 was from a region quite variable in the *pilA* genes of PA14 and PAO1. Overall, *pilA* genes from PA14 and PAO1 were only 53% similar to each other.

We recovered a 3.0-kbp fragment of the *pilABC* gene cluster from PA14 in plasmid pJYP25, and sequence analysis demonstrated that the *pilC* gene in pJY2A and the *pilA* gene in pJY5A were linked in a *pilABC* gene cluster that was otherwise nearly identical to the same cluster in PAO1.

A BLAST search of the insert in pJY15A showed high levels of similarity to the DNA helicase (UvrD) of *C. trachomatis* (27% identity and 45% similarity over 902 bp), the DNA helicase of *Borrelia burgdorferi* (22% identity and 42% similarity over 758 bp), and other DNA helicase family members from other organisms. *P. aeruginosa* strain PAO1 also has *uvrD* in its genome (http://www.pseudomonas.com/GenomeSearch.asp), but there is only 45% similarity between the *uvrD* genes found in *P. aeruginosa* strains PA14 and PAO1. The insert from pJY15A came from an area that was particularly divergent between the sequences of the single *uvrD* genes in each of these two strains.

**Analysis of the *ybtQ* gene homolog in *P. aeruginosa* strain PA14.** DNA sequence analysis of pJY11 showed that the insert was 196 nucleotides in length and had homology to the *ybtQ* gene of *Y. pestis*. This insert was used to recover a 2.1-kbp fragment of *P. aeruginosa* strain PA14 chromosomal DNA overlapping the insert in pJY11, which was cloned into plasmid pJYYBT. Sequencing of pJYYBT revealed a 2,126-bp complete open reading frame, encoding a protein of 585 amino acids with a predicted molecular mass of 63,549 Da and pI of 9.57 (Fig. 3). BLAST search revealed that the highest homologies of this open reading frame were with the ABC transporter protein, YbtQ, of *Y. pestis* (24% identity and 38% similarity in a 1,061-bp overlap), the inner membrane ABC transporter, Irp7, of *Yersinia enterocolitica* (24% identity and 38% similarity over 962 bp), the ABC transporter protein, YbtP, of *Y. pestis* (21% identity and 36% similarity over 1,061 bp), and the ABC transporter protein, Irp6, of *Y. enterocolitica* (21% identity and 36% similarity over 1,061 bp). The genes encoding the Ybt systems of *Y. pestis* and *Y. enterocolitica* show >97% sequence identity (7, 19, 20, 47, 51), and *ybtP* and *ybtQ* in *Y. pestis* are orthologs of *irp6* and *irp7* in *Y. enterocolitica*. Similarity analyses of deduced amino acid sequences by the WU-Blast2 program in EMBL also revealed that the amino acid sequence of the protein encoded in pJYYBT showed 30.7, 31.2, 28.6, and 28.8% similarity to YbtQ, Irp7, YbtP, and Irp6, respectively.

The N-terminal region of the YbtQ protein in *P. aeruginosa* PA14 was less homologous to YbtQ in *Y. pestis* than was the C-terminal region, which is hypothesized to act as a signal sensor (Fig. 4). Analysis of the deduced amino acid sequence of the open reading frame in pJYYBT by ExPASy suggested an amino-terminal hydrophobic region with six possible transmembrane segments, as well as an ABC transporter signature motif in the carboxy-terminal portion of the protein (Fig. 3) (1, 22, 28).

Nucleotide sequence comparison with the genome sequence database of PAO1 (http://www.pseudomonas.com/GenomeSearch.asp) revealed no sequences corresponding to the *ybtQ* homolog in PA14, suggesting that the entire *ybtQ* gene (and possibly surrounding sequences) is uniquely present in *P. aeruginosa* strain PA14 and absent from PAO1. We compared the *ybtQ* gene sequence from PA14 with the sequence of a recently described pathogenicity island, PAGI, present in the majority of pathogenic isolates of *P. aeruginosa* (27) but did not find any sequences homologous to *ybtQ* in PAGI.

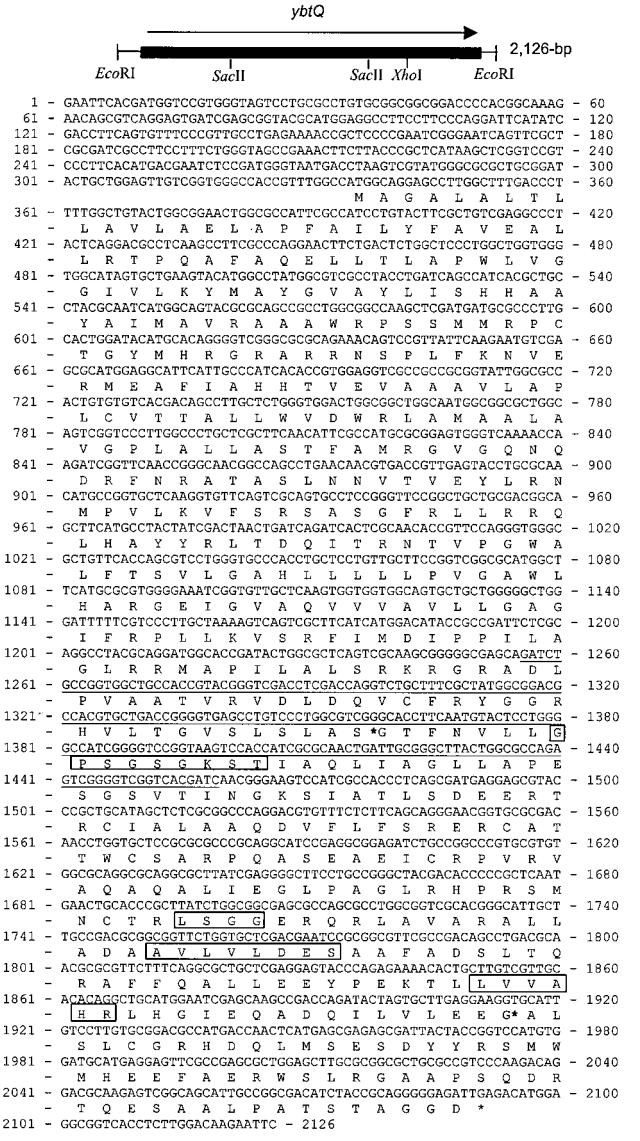


FIG. 3. Map and nucleotide sequence of *ybtQ* gene from *P. aeruginosa* strain PA14. The deduced protein sequence is shown below the coding region of *ybtQ*. Numbering refers to the letter of the nucleotide sequence. The 196-bp of the RDA product recovered in pJY11 is underlined. The asterisks bracket the ABC transporter domain, and the four boxed regions are conserved motifs of the ABC transporter domain (Walker A, ABC signature, Walker B, and an unnamed fourth motif), as defined by Linton and Higgins (28).

**Construction of mutations in the *pilC*, *pilA*, *uvrD*, and *ybtQ* genes of PA14 and determination of phenotypes.** We used standard methods to insertionally inactivate the *pilC*, *pilA*, *uvrD*, and *ybtQ* genes of *P. aeruginosa* strain PA14. Plasmids pJY2M, pJY5M, pJY11M, and pJY15M, encoding 5′- and 3′-truncated *pilC*, *pilA*, *uvrD*, and *ybtQ*, respectively, were integrated into the genome of *P. aeruginosa* strain PA14 by single

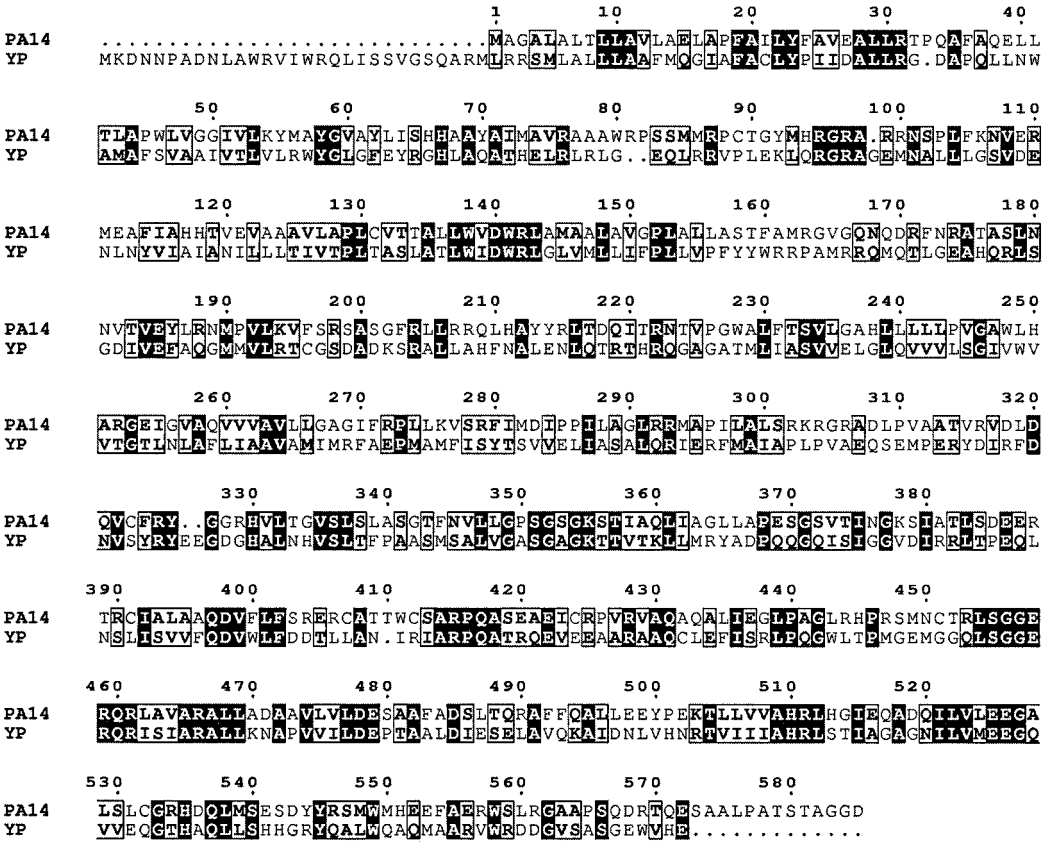


FIG. 4. Alignment of YbtQ homolog in *P. aeruginosa* strain PA14 with YbtQ of *Y. pestis*. An alignment of the YbtQ homolog of *P. aeruginosa* PA14 with YbtQ of *Y. pestis* (YP) was generated with Clustal W. Dark boxes indicate identical residues and light boxes enclose similar residues. Numbers above each pair of sequences reflect the deduced protein sequence of the YbtQ homolog in *P. aeruginosa* PA14.

homologous recombination events. The insertional disruption of the appropriate gene in the corresponding mutant was confirmed by Southern blot analysis.

**Virulence testing of the four mutants in both nonvertebrate and mouse models of infection.** The strains PA14 and PAO1 show differences in a number of models of virulence, including the slow killing of *C. elegans*, killing of wax moth caterpillars, and a burned mouse model of sepsis; we wished to determine if the differences in virulence of these two *P. aeruginosa* strains in these various models related to the genetic differences uncovered by RDA. We therefore compared strains PA14, PAO1, and PA14 mutated in *pilC*, *pilA*, *uvrD*, and *ybtQ* in each of these virulence assays. In the *C. elegans* slow-killing model, there were no differences in virulence between strain PA14 and the mutants JY2M, JY5M, JY11M, and JY15M (data not shown). However, when we determined the $LD_{50}$ of PA14, PAO1, and the four mutants in *G. mellonella*, the *uvrD* and *ybtQ* mutants of PA14 exhibited attenuated virulence, very similar to that of PAO1 (Fig. 5A). The $LD_{50}$s for *G. mellonella* for these three strains were all approximately five-fold higher than for PA14 or the *pilA* or *pilC* mutants ($P < 0.05$).

Rahme et al. (44) reported that *P. aeruginosa* strain PA14 is more virulent in a burned mouse model of sepsis than PAO1, and we confirmed this observation (Fig. 5B). When we examined virulence of the four mutants of PA14 in this model, the *ybtQ* mutant was significantly attenuated, comparable to the attenuation seen with PAO1. Thus, a mutation of *ybtQ*, a gene missing in strain PAO1 (and identified by RDA), in *P. aeruginosa* strain PA14 significantly attenuates the virulence of strain PA14 in two different model systems of infection, to levels comparable to that seen with PAO1.

In *Yersinia*, YbtP and YbtQ are involved in iron acquisition from the environment. We examined the ability of the *ybtQ* mutant of PA14 (strain JY11), to grow in iron-chelated syncase medium. However, we detected no differences between growth of the mutant in this medium and that of wild-type strain PA14, perhaps reflecting the many other iron acquisition systems present in *P. aeruginosa* (data not shown).

## DISCUSSION

In this study, we examined the hypothesis that differences in pathogenesis between specific strains of *P. aeruginosa* may reflect discrete genomic differences identifiable by RDA. Recently, RDA has been used to detect differences between virulent and avirulent *Mycobacterium bovis* strains (31) and between *N. gonorrhoeae* and *Neisseria meningitidis* (60). Previous studies have demonstrated that *P. aeruginosa* strain PA14 is significantly more virulent in a slow killing assay with *C. elegans* (58) and in a burned mouse model of infection (45)

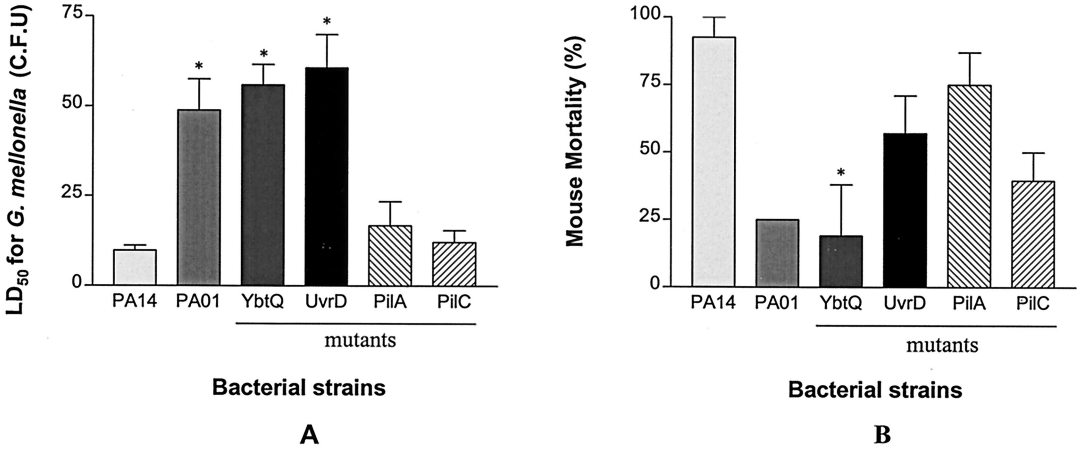


FIG. 5. Evaluation of mutants for virulence defects in wax moths and mice. (A) $LD_{50}$s of *P. aeruginosa* strains PA14 and PAO1 and four mutants in fifth-instar *G. mellonella* larvae. Ten larvae were injected at each dilution (containing 0 to $10^6$ bacteria), and larvae were scored as live or dead after 60 h at 25°C. The data are the means and standard deviations of three independent experiments. Asterisks indicate statistically significant differences from *P. aeruginosa* strain PA14. (B) Mortality in a burned mouse model for *P. aeruginosa* strains PA14 and PAO1 and for four mutants. Eight mice per experiment were injected subcutaneously with $5 \times 10^5$ CFU of each *P. aeruginosa* strain, and the number of animals that died as a result of sepsis was monitored each day for 10 days. The data are the means and standard deviations of two independent experiments for PA14 and the four mutants; PAO1 was tested once. The asterisk indicates a statistically significant difference from PA14.

than the well-characterized and sequenced strain PAO1. We utilized these differences in pathogenesis, in combination with RDA, to examine specific genes in strain PA14 that might underlie differences in pathogenesis from strain PAO1. We identified differences in the *pilC*, *pilA*, and *uvrD* genes between *P. aeruginosa* strains PA14 and PAO1 and identified a *ybtQ* homolog in strain PA14 that was entirely missing in strain PAO1. We constructed mutations in each of these four genes and compared the pathogenesis of these mutants with that of the parent, PA14, and the reference strain, PAO1, in both nonvertebrate model systems and a mouse model of burn infection.

Mutations of the *pilC* and *pilA* genes in strain PA14 did not alter the virulence of the strains in the various model systems tested. The *pilC* and *pilA* genes in *P. aeruginosa* strain PA14 were located in the same *pilABC* cluster as in strain PAO1 but were divergent at the sequence level; additional homologs of *pilA* and *pilC* were not identified in *P. aeruginosa* strain PA14 by Southern blotting (data not shown). When the deduced amino acid sequence of PilC from *P. aeruginosa* strain PA14 was compared with that from strain PAO1, the proteins were shown to have very high homology in a number of conserved domains, separated by a number of variable regions. This structure is reminiscent of the PilE protein in *N. gonorrhoeae*, which has been shown to undergo pilus antigenic variation. This antigenic variation occurs by the high-frequency, unidirectional transfer of DNA sequences from one of several silent pilin loci (*pilS* genes) into the expressed pilin gene locus (*pilE*), resulting in changes in the primary pilin protein sequence (5, 35, 56, 57). Since we did not detect any additional *pilC* homologs in *P. aeruginosa* by Southern blot, the mechanism of divergence between the *pilC* genes in *P. aeruginosa* strains PA14 and PAO1 is currently uncertain.

The PA14 *uvrD* mutant was attenuated in virulence in *G. mellonella* but not in *C. elegans* or the burned mouse model, whereas the *ybtQ* mutant was attenuated in virulence in both *G. mellonella* and the burned mouse model (but not in *C. elegans*). Previous studies of *P. aeruginosa* mutants that were tested in both *C. elegans* and *G. mellonella* have shown that several mutants exhibit attenuated pathogenesis specifically in one host but not the other (25, 58, 59). This suggests that screening for pathogenesis in a variety of different hosts may lead to the identification of subsets of virulence factors that are critical for infections in specific hosts, as well as others important across hosts. For example, Mahajan-Miklos et al. (32) have suggested that screening for mutants of *P. aeruginosa* attenuated in a fast-killing assay of *C. elegans* under hyperosmolar and low-pH conditions may identify genes that are also important for virulence in a cystic fibrosis lung infection model.

*P. aeruginosa* utilizes several siderophores for high-affinity iron uptake, including pyoverdin and pyochelin (10, 11, 43). Pyoverdin production has been previously shown to be required for bacterial colonization of the lung in a rat infection model (43) and to correlate with lethality in a burned mouse infection model (34). Pyochelin has been shown to promote bacterial growth and lethality when injected into the peritoneal cavities of mice simultaneously with an avirulent mutant of *P. aeruginosa* strain PAO1, isolated by repetitive passage in mice (9).

Yersiniabactin, a phenolate-thiazole siderophore, was first purified from *Y. enterocolitica* and subsequently from *Y. pestis*. In *Y. pestis*, yersiniabactin may play a role in establishing infection at the site of a flea bite, as mutants that are unable to produce or transport yersiniabactin are avirulent in mice by peripheral routes of infection but fully virulent when infected intravenously (4, 61). Several of the genes needed for the biosynthesis and utilization of yersiniabactin are clustered in a pathogenicity island that is part of an unstable region on the *Y. pestis* chromosome, the *pgm* locus (4, 14, 19, 20, 40). All highly pathogenic species of *Yersinia* have similar genes clustered on a high-pathogenicity island.

Our results suggests that the *ybtQ* homolog that is present in

strain PA14 but absent in strain PAO1 may at least partially explain the differences in pathogenesis of these two strains for the burned mouse model as well as for *G. mellonella*. One possibility is that *P. aeruginosa* strain PA14 contains a pathogenicity island encoding the *ybtQ* homolog and other genes absent from strain PAO1 and acquired by horizontal gene transfer. Extensive genomic rearrangements, as well as acquisition and loss of large blocks of DNA, have been previously demonstrated in different *P. aeruginosa* isolates (49). We have not yet recovered sufficient DNA flanking the *ybtQ* homolog to further examine the possibility of a pathogenicity island; such experiments are under way.

The role of the *ybtQ* homolog in the pathogenesis of *P. aeruginosa* strain PA14 for *G. mellonella* and mice is also uncertain. We were not able to demonstrate impaired growth under iron-limited conditions by the *ybtQ* mutant, but this may relate to the multiple siderophore systems available for use by *P. aeruginosa* during in vitro growth. Further experiments to examine the relationship between this gene, iron uptake, and pathogenesis of *P. aeruginosa* strain PA14 in various model systems are also under way.

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