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A Cystic Fibrosis Epidemic Strain of *Pseudomonas aeruginosa* Displays Enhanced Virulence and Antimicrobial Resistance

Prabhakar Salunkhe,[1]† Catherine H. M. Smart,[2] J. Alun W. Morgan,[3] Stavroula Panagea,[2] Martin J. Walshaw,[4] C. Anthony Hart,[2] Robert Geffers,[5] Burkhard Tümmler,[1] and Craig Winstanley[2]*

Clinical Research Group OE 6711, Medical Research School Hannover, 30625 Hannover, Germany[1]; Division of Medical Microbiology, University of Liverpool, Liverpool L69 3GA, United Kingdom[2]; Warwick-HRI, University of Warwick, Wellesbourne, Warwickshire CV35 9EF, United Kingdom[3]; Regional Adult Cystic Fibrosis Unit, Cardiothoracic Center, Liverpool L14 3PE, United Kingdom[4]; and German Research Center for Biotechnology, Mascheroder Weg 1, 38124-Braunschweig, Germany[5]

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The Liverpool epidemic strain (LES) of *Pseudomonas aeruginosa* is a transmissible aggressive pathogen of cystic fibrosis (CF) patients. We compared transcriptome profiles of two LES isolates with each other and with a laboratory and genetic reference strain (PAO1) after growth to late exponential phase and following exposure to oxidative stress. Both LES isolates exhibited enhanced antimicrobial resistances linked to specific mutations in efflux pump genes. Although transcription of AmpC β-lactamase was up-regulated in both, one LES isolate contained a specific mutation rendering the *ampC* gene untranslatable. The virulence-related quorum-sensing (QS) regulon of LES431, an isolate that caused pneumonia in the non-CF parent of a CF patient, was considerably up-regulated in comparison to either isolate LES400, associated with a chronic CF infection, or strain PAO1. Premature activation of QS genes was detected in isolates from both non-CF parents and the CF patient in a previously reported infection episode. LES isolates lacking the up-regulated QS phenotype contained different frameshift mutations in *lasR*. When fed to *Drosophila melanogaster*, isolate LES431 killed the fruit flies more readily than either isolate LES400 or strain PAO1, indicating that virulence varies intraclonally. The LES may represent a clone with enhanced virulence and antimicrobial resistance characteristics that can vary or are lost due to mutations during long-term colonization but have contributed to the successful spread of the lineage throughout the CF population of the United Kingdom.

Pseudomonas aeruginosa, the most common pathogen associated with morbidity and mortality in cystic fibrosis (CF), causes chronic lung infections that once established are impossible to eradicate. For many years, the prevailing view was that individual CF patients acquired *P. aeruginosa* infections separately and thus carried their own unrelated strains. In 1996, Cheng et al. (6) reported the spread of a drug-resistant strain of *P. aeruginosa* among patients in a children's CF center in Liverpool. Since then, there have been other reports of CF "epidemic" strains (14, 45). An analysis of post-2000 patient samples has indicated that of 80 CF patients (from a total of 92 sampled) in the Liverpool adult center colonized with *P. aeruginosa*, 79% carry the Liverpool epidemic strain (LES) (35). In a recent study (45) involving over 1,200 isolates from 31 CF centers in England and Wales, the LES was identified as the most common clone, present in 48% of CF centers and accounting for 11% of the isolates. The LES appears to be more aggressive than other strains of *P. aeruginosa*. It has been able to replace previously established strains of *P. aeruginosa* (superinfection) (28) and has infected the non-CF parents of a CF patient, causing significant morbidity (29). Furthermore, there is greater morbidity among CF patients colonized with the LES than among those carrying nonepidemic strains of *P. aeruginosa* (1). Clearly, this strain represents a transmissible and aggressive clone well adapted to the CF environment.

The genome of *P. aeruginosa* displays a mosaic structure, with all strains possessing a highly conserved backbone comprising the vast majority of their genome and including the recognized virulence factors (9, 56). Variations between strains include the presence or absence of genomic islands, and there is some evidence to suggest that the LES possesses such islands (36). However, variations in gene expression have been shown to influence the virulence of an autoaggressive and highly adherent small-colony variant of a CF isolate, with significant up-regulation of virulence-related genes occurring (51). The success of the LES may be due either to the prior acquisition of genes or islands, to transcriptional variations in gene expression, or to a combination of both. We hypothesize that such changes contribute to greater colonization and/or transmissibility of the strain, enhancing its ability to cause CF infections, and may lead to enhanced virulence manifesting itself in episodes such as the infection of non-CF parents.

To test the hypothesis that the additional properties of the LES may be due to variations in gene expression, we compared the transcriptional signature of two LES isolates (associated with chronic and acute infections, respectively) to each other and to the laboratory and genetic reference strain PAO1 under two different growth conditions designed to maximize the number of

* Corresponding author. Mailing address: Division of Medical Microbiology, University of Liverpool, Duncan Building, Daulby St., Liverpool L69 3GA, United Kingdom. Phone: 44 151 706 4388. Fax: 44 151 706 5805. E-mail: C.Winstanley@liv.ac.uk.

† Present address: Department of Microbial Pathogenesis and Vaccine Research, German Research Centre for Biotechnology, Mascheroder Weg 1, 38124 Braunschweig, Germany.

expressed genes: late exponential phase and stationary phase with exposure to hydrogen peroxide. Here, we report variations in the expression of virulence-related genes not only between LES isolates and strain PAO1 but between the two LES isolates.

MATERIALS AND METHODS

Bacterial strains. Isolate LES400 is our laboratory reference isolate for the LES, used in previous genetic analysis (36), and was isolated from a CF patient who had been colonized for at least 6 months (chronic infection). It has an identical pulsed-field gel electrophoresis (PFGE) pattern to the earliest-known LES isolate from 1988. The other LES isolates were sputum culture isolates taken from a case involving respiratory tract infection of the non-CF parents of a CF patient (29). Isolates LES431 and LES430 were both isolated from the CF patient's father, who presented with pneumonia. Isolate LES417 was isolated from the patient's mother, who presented with pyrexia and bilateral chest wheezes. Isolates LES416 and LESB44 were isolated from the CF patient. Isolates LES416, LES417, LES430, and LES431 share an identical PFGE pattern. Strain PAO1 is a widely studied laboratory strain of *P. aeruginosa* for which the entire genome sequence is known (47). PFGE analysis of SpeI-digested genomic DNA was carried out using 1% (wt/vol) agarose gels and interpreted according to the protocol of Tenover et al. (49).

Growth conditions and RNA isolation for microarray analysis. In the absence of stress, *P. aeruginosa* strains were grown up to late exponential phase (optical density at 600 nm [OD_{600}] of 2.7 to 3.0) in Luria broth (LB). For exposure of bacteria to hydrogen peroxide, stationary phase-grown cultures (3×10^{10} cells) were resuspended in fresh LB and kept in a dialysis tube (14-kDa cutoff; 25 mm) with an effective length of 6 cm for the exchange of fluids. Then, the dialysis tube was resuspended in a 1-liter Erlenmeyer flask containing 600 ml of LB with or without 10 mM hydrogen peroxide (Sigma-Aldrich) to generate oxidative stress. The flasks were incubated at 37°C and 200 rpm on a rotary shaker for 2 h.

Total RNA was extracted from approximately 3×10^{10} cells by a modified hot-phenol method (48). The procedure for RNA isolation, purification, and quantification has been described previously (51). For each GeneChip experiment, the culturing of bacteria, exposure to hydrogen peroxide or LB control, and subsequent RNA isolation were performed in triplicate on the same day. Equal amounts of each of three preparations were then pooled to a total of 10 μg for cDNA synthesis and hybridization onto a single GeneChip. This procedure was duplicated. Thus, ultimately, two GeneChips per strain and growth condition were scanned at 570 nm with 3-μm resolution by the Affymetrix scanner.

GeneChip microarray analysis. The generation of cDNA and subsequent biotin-ddUTP terminal-labeling steps were performed as described in the manufacturer's instructions for the *P. aeruginosa* GeneChip (Affymetrix), using the 10 μg of total RNA mixed with random primers (Invitrogen) and control in vitro transcripts of 10 non-*Pseudomonas* gene sequences (kindly provided by S. Lory and coworkers, University of Washington), as described previously (51). GeneChip hybridization and washing were carried out following the manufacturer's instructions (Affymetrix) and as described previously (51).

The *P. aeruginosa* PAO1 GeneChip contains oligonucleotide probes for 5,549 protein-coding genes, 18 tRNA genes, a representative rRNA cluster, and 199 intergenic regions selected from the annotated genome of *P. aeruginosa* strain PAO1 (47). In addition, there are probes for 117 genes from *P. aeruginosa* strains other than PAO1 and 14 genes from other species, which can serve as controls (31). Data analysis was performed using the Affymetrix Microarray Suite software (version 5.0) with Affymetrix default parameters. The average microarray hybridization signal intensity was scaled to 150. Two GeneChips for each strain per condition were compared by the four-comparison survival method (3, 5) as follows. The data were imported into a Microsoft Access database capable of searching for genes that significantly changed their signal intensities by the Wilcoxon rank test, with a minimum of a twofold change in all four comparisons. The arithmetic average and the standard deviation of the four comparisons were calculated. As an independent criterion for significantly changed signal intensities, a Bonferroni correction of the signal ratios obtained from the MicroArray Suite software was applied to account for the number of tests (40), which in this case was the total number of open reading frames (ORFs) on the chip. First, the ratio of calibrated and corrected hybridization signals per gene (S_i) obtained from cultures grown under identical conditions was verified to follow a Gaussian distribution, and the variance (σ) was calculated. mRNA transcript levels of a gene (i) were considered to be significantly differentially expressed, if the ratio $S(i)_A/S(i)_B$ or $S(i)_B/S(i)_A$ exceeds the threshold ($1 + u\sigma$), whereby the factor u defines that upper boundary of the normalized Gaussian integral $\Phi(u)$ where $\Phi(u) = x^n$ matches the Bonferroni-corrected 95% confidence interval in the expression $(1 - \alpha) = x^n$ (here, n = 5,900, $\alpha = 0.025$, and $0.975 \ll x < 1.0$).

In summary, changes were only classified as significant if they fulfilled the criteria of the four-comparison survival method and exceeded the threshold of the Bonferroni correction for multiple testing. Data were combined with the latest annotation (15 December 2004) from the website of the *P. aeruginosa* PAO1 sequence and the community annotation project provided at http://www.pseudomonas.com.

Generation of targets from genomic DNA hybridization. For hybridization of genomic DNA from isolates LES400 and LES431 on the PAO1 GeneChip, 25 μg of genomic DNA from stationary-phase-grown cells was fragmented with 7.5 U of DNase I (Amersham) at 37°C for 10 min. This enzyme produced a majority of fragments in the range of 50 to 200 bp, which is suitable for GeneChip hybridization. The fragmented DNA from two independent genomic DNA preparations for each LES isolate was denatured at 95°C, labeled, and then hybridized on a GeneChip as described for the cDNA expression analysis. The absence or presence of genes was classified as described previously (56).

Exoproduct secretion assays. All cultures were inoculated in either King's A medium or in LB to an OD_{600} of approximately 0.05 and then incubated at 37°C with shaking (300 rpm). The amount of pyocyanin in culture supernatants was quantified by measuring the OD_{695} value (16). LasA protease and elastase activities were measured by determination of the ability of *P. aeruginosa* culture supernatants to lyse boiled *Staphylococcus aureus* cells, leading to a decrease in OD_{600} (15), and by the elastin Congo red assay (38), respectively. In the latter assay, insoluble elastin Congo red was removed by centrifugation, and the increase in the OD_{495} value in supernatants was used as a measure of elastase activity.

Antimicrobial sensitivity tests and β-lactamase activities. MICs for antimicrobial agents were determined using E-test strips according to the manufacturer's instructions (AB Biodisk).

PCR amplification and nucleotide sequencing. Details of the oligonucleotide primers (Sigma-Genosys) used in PCR assays and for nucleotide sequencing will be made available on request. PCR amplicons were purified using S-400 microspin columns (Amersham-Pharmacia Biotech) and sequenced by Lark Technologies, Inc., using the same oligonucleotide primers employed in the PCR amplification and internal primers.

Virulence against fruit flies. Virulence against *Drosophila melanogaster* was assessed essentially as described by Chugani et al. (8). The fruit flies were obtained from Blades Biological, Ltd. (Cowden, Kent, United Kingdom), and three independent assays were run consecutively.

RESULTS AND DISCUSSION

Whole-genome comparisons. PFGE of the three isolates used in the microarray analysis indicated that the LES isolates shared few bands in common with strain PAO1 and differed from each other by two bands (Fig. 1). Size estimations using PFGE gels indicated that the LES did not have a larger genome than strain PAO1.

GeneChip analysis using DNA from isolates LES400 and LES431 indicated the presence in LES strains of at least 95% of PAO1 genes and confirmed the additional presence of O6 antigen genes and PAGI-1 genes, as reported previously (36). In accordance with the known pyoverdine type of the LES (type III) (36), PAO1 pyoverdine sythesis and receptor genes were identified as absent from LES isolates. Among other absent genes/gene clusters were a transport-related cluster (PA0202 to PA0206), two bacteriophage-related clusters (PAO632 to PA0648 and PA0715 to PA0759) and a lipopolysaccharide-related cluster (PA3142 to PA3160). Also absent from LES isolates was the putative polysaccharide-related cluster PA1378 to PA1393, a cluster of type IV pilus genes (PA4525 to PA4527; *pilABC*), and several other clusters of unknown function (PA0980 to PA0985, PA2100 to PA2106, PA2218 to PA2228, PA2730 to PA2736, and PA3500 to PA3513). PCR assays targeting PA0203, PA0641, and PA1384 (*galE*) supported the notion that these genes were absent from

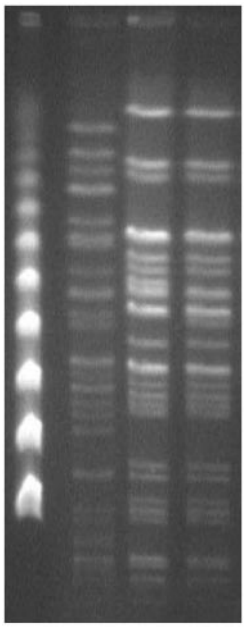

FIG. 1. PFGE comparisons of LES isolates and strain PAO1. M, pulse marker, 50 to 1,000 kb (Sigma-Aldrich); lane 1, PAO1; lane 2, LES400; lane 3, LES431.

TABLE 1. Numbers of differentially expressed genes

Growth condition and change[b]	No. of expressed genes[a]		
	LES400 vs PAO1	LES431 vs PAO1	LES431 vs LES400
LB up	222	225	211
QS activated	8	127	142
QS repressed	5	6	4
Antimicrobial	12	8	5
LB down	119 (137)	103 (112)	137
QS activated	10	6	1
QS repressed	11	9	3
Antimicrobial	1	1	3
H_2O_2 up	146	349	219
QS activated	18	70	6
QS repressed	1	3	0
Antimicrobial	5	12	3
H_2O_2 down	350 (367)	251 (299)	90
QS activated	8	9	11
QS repressed	10	21	5
Antimicrobial	0	1	3

[a] Values for down-regulated genes when LES isolates were compared to strain PAO1 were adjusted by subtracting genes that were recorded as absent from LES isolates. The figure prior to this adjustment is shown in parentheses.

[b] Up- or down-regulation under either growth condition (LB or H_2O_2 exposed) was at least twofold. QS, number of quorum-sensing regulated genes previously reported as either QS activated or QS repressed (43, 51); antimicrobial, genes associated with antimicrobial susceptibility.

the LES isolates. Variations in the *pilABC* cluster between strains of *P. aeruginosa* have been reported previously (7, 18). Initially, PCR amplification assays using primers designed to the PA4526 (*pilB*) sequence of strain PAO1 were negative for both LES isolates. However, when primers designed on the basis of conservation between strains PAO1 and PA14 were used (PILBF2 and PILBR2), isolates LES400 and LES431 were PCR positive for *pilB*, suggesting that the *pil* genes are present in LES isolates but vary in sequence from their equivalents in strain PAO1.

Although PAGI-1 coding sequences 1 to 31 were detected in the LES isolates, coding sequences 34 to 51 were lacking. This observation was supported by PCR assays for one of the missing genes, suggesting that the LES carries a truncated version of the PAGI-1 island, lacking the region with a G+C content significantly below that of the genome average (23). This island is also only partially present in the other *P. aeruginosa* strain that has been genome sequenced (PA14). Only minor differences in gene content were detected between the two LES isolates.

Global GeneChip expression analysis. Gene expression microarrays were used to compare the transcriptomes of the two LES isolates against strain PAO1 and against each other, following growth to late exponential phase in LB and after exposure to hydrogen peroxide. Tables listing all those genes identified as up- or down-regulated are available at C. Winstanley's website (http://www.liv.ac.uk/mmgum/). A summary of the numbers of differentially expressed genes in the various comparisons is shown in Table 1. The following text serves to highlight some of the main features of the expression analysis.

The most striking finding was that in isolate LES431 grown under LB growth conditions and compared to either strain PAO1 or isolate LES400, the majority (56% and 67% for strain PAO1 and isolate LES400, respectively) of up-regulated genes were among those reported previously as activated by quorum sensing (QS) (Tables 1 to 3). Thus, isolate LES431 expressed substantially elevated levels of mRNA transcripts for many of the known *P. aeruginosa* virulence genes (44, 52), including those encoding alkaline protease, elastase, LasA protease, Clp proteases, CbpD, phenazine biosynthesis, hydrogen cyanide synthesis, aminopeptidase, and lectin. Notably, mRNA levels were elevated to similar ratios whether compared to another member of the same clone (isolate LES400) or to a member of another clone (strain PAO1).

Among the genes up-regulated in isolates LES400 and LES431 compared to strain PAO1 under both growth conditions were several associated with antimicrobial susceptibility, including the *ampC* β-lactamase gene, the MexAB-OprM and MexXY efflux pumps (Table 4), and the pyochelin biosynthesis genes (PA4221 to PA4231). Regulatory genes associated with alginate production (*algU* and *mucA*) were up-regulated following growth in LB only in isolate LES400. Notable genes up-regulated in isolate LES431 following oxidative stress compared to either strain PAO1 or isolate LES400 included a bacteriophage-related gene cluster (PA0611to PA0628).

Genes down-regulated in both LES isolates compared to strain PAO1 under both growth conditions included genes from clusters involved in anaerobic metabolism (*nirJLFCMSQ* [PA0511 to PA0520], *norBC* [PA0523 to PA0524], *nosRZDF* [PA3391 to PA3394], and *arcDABC* [PA5170 to PA5173]), motility/chemotaxis (*flgBCDEGJK* [PA1077 to PA1086], *fliCD* [PA1092 to PA1096], and *pctCAB* [4307 to 4310]), and twitching motility (*pilGIJK* [PA0408 to PA0412]). Type IV pilus-

TABLE 2. Summary of quorum sensing-related genes that were up-regulated in (i) isolate LES400 compared to strain PAO1, (ii) isolate LES431 compared to strain PAO1, and (iii) isolate LES431 compared to isolate LES400

ORF[a]	Gene name	LB LES400 ↑ vs PAO1	H_2O_2 LES400 ↑ vs PAO1	LB LES431 ↑ vs PAO1	H_2O_2 LES431 ↑ vs PAO1	LB LES431 ↑ vs LES400	H_2O_2 LES431 ↑ vs LES400	Product description
QS activated								
PA0026				2.6		11		Hypothetical protein
PA0050					3.3			Hypothetical protein
PA0059	*osmC*			4.6		6.3		Osmotically inducible protein OsmC
PA0106	*coxA*		64			26		Cytochrome *c* oxidase, subunit 1
PA0107			24	21		5.0		Conserved hypothetical protein
PA0122				15		14		Conserved hypothetical protein
PA0143	*nuh*			11		24		Nonspecific ribonucleoside hydrolase
PA0176						3.1		Probable chemotaxis transducer
PA0355	*pfpI*					2.8		Protease PfpI
PA0364		5.4		4.6				Probable oxidoreductase
PA0366		4.0		3.3				Probable aldehyde dehydrogenase
PA0447	*gcdH*				2.4			Glutaryl-CoA dehydrogenase
PA0567				16		9.2		Conserved hypothetical protein
PA0586			4.3			8.2		Conserved hypothetical protein
PA0588				5.2		6.9		Conserved hypothetical protein
PA0852	*cbpD*			44		55		Chitin-binding protein CbpD precursor
PA0996	*pqsA*			3.5	9.3	16		Probable coenzyme A ligase
PA0997	*pqsB*			6.5	5.8	7.3		Homologous to β-ketoacyl-acyl carrier protein synthase
PA0998	*pqsC*			4.6	6.5	5.0		Homologous to β-ketoacyl-acyl carrier protein synthase
PA0999	*pqsD*			5.3	5.3	6.5		3-Oxoacyl-acyl carrier protein synthase III
PA1000	*pqsE*				6.2			Quinolone signal response protein
PA1001	*phnA*			12.1	4.6			Anthranilate synthase component I
PA1173	*napB*					5.6		Cytochrome *c*-type protein NapB precursor
PA1174	*napA*			3.5		4.4		Periplasmic nitrate reductase protein NapA
PA1175	*napD*			3.0		5.0		NapD protein of periplasmic nitrate reductase
PA1176	*napF*					6.4		Ferredoxin protein NapF
PA1177	*napE*			5.8		4.4		Periplasmic nitrate reductase protein NapE
PA1247	*aprE*					7.5		Alkaline protease secretion protein AprE
PA1248	*aprF*					12		Alkaline protease secretion outer membrane protein AprF precursor
PA1250	*aprI*			16		7.5		Alkaline proteinase inhibitor AprI
PA1323				11		6.7		Hypothetical protein
PA1324			7.5	5.3	3.3	5.8		Hypothetical protein
PA1404			16.9	4.1	3.8	3.6		Hypothetical protein
PA1431	*rsaL*			20	14	655	16	Regulatory protein RsaL
PA1432	*lasI*					21		Autoinducer synthesis protein LasI
PA1656				3.3				Hypothetical protein
PA1657				14		11		Conserved hypothetical protein
PA1658				4.4		24		Conserved hypothetical protein
PA1660						11		Hypothetical protein
PA1662				15				Probable ClpA/B-type protease
PA1667				12				Hypothetical protein
PA1784				3.6	3.6	5.9		Hypothetical protein
PA1869				78	28	68		Probable acyl carrier protein
PA1871	*lasA*			4.9	5.2	12		LasA protease precursor
PA1874				5.3				Hypothetical protein
PA1881				31		3.8		Probable oxidoreductase
PA1891					5.9			Hypothetical protein
PA1893					6.2			Hypothetical protein
PA1894				3.4	11	4.8	6.1	Hypothetical protein
PA1895					8.9	3.8		Hypothetical protein
PA1896						11		Hypothetical protein
PA1897				4.2	13	24		Hypothetical protein
PA1901	*phzC2*			38		126		Phenazine biosynthesis protein PhzC
PA1902	*phzD2*			49		77		Phenazine biosynthesis protein PhzD
PA1903	*phzE2*			31		35		Phenazine biosynthesis protein PhzE
PA1904	*phzF2*			86		91		Probable phenazine biosynthesis protein
PA1905	*phzG2*			22		70		Probable pyridoxamine 5′-phosphate oxidase
PA1999				2.8		2.6		Probable CoA transferase, subunit A
PA2000				2.9		2.8		Probable CoA transferase, subunit B
PA2001	*atoB*			3.5		2.5		Acetyl-CoA acetyltransferase
PA2030				15		10		Hypothetical protein
PA2031				6.2		10		Hypothetical protein
PA2066				6.0		4.4		Hypothetical protein

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TABLE 2—*Continued*

ORF[a]	Gene name	LB LES400 ↑ vs PAO1	H_2O_2 LES400 ↑ vs PAO1	LB LES431 ↑ vs PAO1	H_2O_2 LES431 ↑ vs PAO1	LB LES431 ↑ vs LES400	H_2O_2 LES431 ↑ vs LES400	Product description
PA2067				8.5		11		Probable hydrolase
PA2068				20		18		Probable MFS transporter
PA2069				25		45		Probable carbamoyl transferase
PA2080						5.4		Hypothetical protein
PA2143				33				Hypothetical protein
PA2146			29	13	18	14		Conserved hypothetical protein
PA2157					4.1			Hypothetical protein
PA2159			13	3.8		12		Conserved hypothetical protein
PA2165				5.4		5.0		Probable glycogen synthase
PA2166		6.5		117	7.0	18		Hypothetical protein
PA2171				14	5.4	12		Hypothetical protein
PA2172				6.5	3.2			Hypothetical protein
PA2180					2.3			Hypothetical protein
PA2190				16		25		Conserved hypothetical protein
PA2193	*hcnA*			58		35		Hydrogen cyanide synthase HcnA
PA2194	*hcnB*			15		111		Hydrogen cyanide synthase HcnB
PA2195	*hcnC*			12		40		Hydrogen cyanide synthase HcnC
PA2274				4.1	2.6	6.7		Hypothetical protein
PA2300	*chiC*			7.7	7.0			Chitinase
PA2305				9.4		23		Probable nonribosomal peptide synthetase
PA2328					4.3			Hypothetical protein
PA2329					4.9			Probable ATP-binding component of ABC transporter
PA2330					2.5			Hypothetical protein
PA2331					3.6			Hypothetical protein
PA2345					2.8			Conserved hypothetical protein
PA2365			32	12		7.3		Conserved hypothetical protein
PA2366			13	7.7	6.3	13		Conserved hypothetical protein
PA2367				15		6.2		Hypothetical protein
PA2423						5.6		Hypothetical protein
PA2433			15	6.9	2.9	2.8		Hypothetical protein
PA2512	*antA*	7.6						Anthranilate dioxygenase large subunit
PA2513	*antB*	18		50				Anthranilate dioxygenase small subunit
PA2552						2.2		Probable acyl-CoA dehydrogenase
PA2553						2.7		Probable acyl-CoA thiolase
PA2587	*pqsH*			5.7		28		Probable FAD-dependent mono-oxygenase
PA2588				26		22		Probable transcriptional regulator
PA2591	*vqsR*				6.0	12		Probable transcriptional regulator
PA2592				4.3	5.4	4.9		Probable periplasmic spermidine/putrescine-binding protein
PA2747				6.6		5.4		Hypothetical protein
PA2939				147		134		Probable aminopeptidase
PA3032	*snr1*		10		5.9			Cytochrome *c* Snr1
PA3104	*xcpP*					3.7		Secretion protein XcpP
PA3181		5.4		3.5			10	2-Keto-3-deoxy-6-phosphogluconate aldolase
PA3182	*pgl*	5.7		3.8			4.8	6-Phosphogluconolactonase
PA3183	*zwf*	6.2		2.9			11	Glucose-6-phosphate 1-dehydrogenase
PA3326				14	11	14		Probable Clp family ATP-dependent protease
PA3327					5.6			Probable nonribosomal peptide synthetase
PA3328				6.1	9.1	8.1		Probable FAD-dependent mono-oxygenase
PA3329				37	34	33		Hypothetical protein
PA3330				34	46	55		Probable short-chain dehydrogenase
PA3331				6.1	16	8.6		Cytochrome P450
PA3332				26	30	13		Conserved hypothetical protein
PA3333	*fabH2*			6.6	52	6.0		3-Oxoacyl-acyl carrier protein synthase III
PA3334					20			Probable acyl carrier protein
PA3335					19			Hypothetical protein
PA3347						3.1		Hypothetical protein
PA3361	*lecB*			26		6.0		Fucose-binding lectin PA-IIL
PA3369			9.4	11		6.3		Hypothetical protein
PA3370				5.2		4.8		Hypothetical protein
PA3418	*ldh*		10	4.2		3.3		Leucine dehydrogenase
PA3476	*rhlI*				11			Autoinducer synthesis protein RhlI
PA3477	*rhlR*			6.5	2.7	12		Transcriptional regulator RhlR
PA3478	*rhlB*			12	9.3	57		Rhamnosyltransferase chain B
PA3479	*rhlA*			28	16	46		Rhamnosyltransferase chain A
PA3520				38		9.0		Hypothetical protein
PA3535				10		12		Probable serine protease

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TABLE 2—*Continued*

ORF[a]	Gene name	LB LES400 ↑ vs PAO1	H_2O_2 LES400 ↑ vs PAO1	LB LES431 ↑ vs PAO1	H_2O_2 LES431 ↑ vs PAO1	LB LES431 ↑ vs LES400	H_2O_2 LES431 ↑ vs LES400	Product description
PA3688						4.0		Hypothetical protein
PA3691			11	8.7	2.9	8.5		Hypothetical protein
PA3692			8.2	6.5		7.8		Probable outer membrane protein precursor
PA3724	*lasB*			413	4.0	597		Elastase LasB
PA3888				3.4		2.8		Probable permease of ABC transporter
PA3904				9.9		21		Hypothetical protein
PA3906					9.5	21		Hypothetical protein
PA3907				7.4		24		Hypothetical protein
PA3923				5.9		27		Hypothetical protein
PA4117						3.2		Probable bacteriophytochrome
PA4129					6.7	4.1		Hypothetical protein
PA4130					7.2	3.8		Probable sulfite or nitrite reductase
PA4131					6.6	7.0		Probable iron-sulfur protein
PA4133				3.9	28	16		Cytochrome *c* oxidase subunit (cbb3 type)
PA4134					4.9	42		Hypothetical protein
PA4139				6.9		3.8		Hypothetical protein
PA4141				66	33	102		Hypothetical protein
PA4142				9.6		5.5		Probable secretion protein
PA4171				10		13		Probable protease
PA4175	*prpL*			19		52		Pvds-regulated endoprotease, lysyl class
PA4205	*mexG*			45	8.7	5.9		Hypothetical protein
PA4206	*mexH*			11	6.7	9.2		Probable RND efflux membrane fusion protein precursor
PA4207	*mexI*			15	8.4	6.2	4.9	Probable RND efflux transporter
PA4208	*opmD*			4.3	8.0	5.5		Probable outer membrane protein precursor
PA4209	*phzM*			31		37		Probable phenazine-specific methyltransferase
PA4210	*phzA1*			19		27		Probable phenazine biosynthesis protein
PA4211	*phzB1*			124		525		Probable phenazine biosynthesis protein
PA4217	*phzS*			55		69		Flavin-containing mono-oxygenase
PA4296				4.1		8.1		Probable two-component response regulator
PA4306					9.0			Hypothetical protein
PA4311			13	4.0		2.9		Conserved hypothetical protein
PA4496						3.4		Probable binding protein component of ABC transporter
PA4498						4.6		Probable metallopeptidase
PA4590	*pra*			27		10		Protein activator
PA4648				10		18		Hypothetical protein
PA4649					19			Hypothetical protein
PA4738			7.8	19	5.0	18		Conserved hypothetical protein
PA4739			13	26	8.1	18		Conserved hypothetical protein
PA4778				6.4		6.6		Probable transcriptional regulator
PA4869						5.4		Hypothetical protein
PA4876	*osmE*			8.3		4.6		Osmotically inducible lipoprotein OsmE
PA4880				6.1		8.2		Probable bacterioferritin
PA5058	*phaC2*			3.1		4.1		Poly(3-hydroxyalkanoic acid) synthase 2
PA5061				3.1		2.9		Conserved hypothetical protein
PA5220				26		21		Hypothetical protein
PA5481				38	8.7	29		Hypothetical protein
PA5482				19	12	29		Hypothetical protein
QS repressed								
PA0887	*acsA*					3.5		Acetyl-coenzymeA synthetase
PA1559		15			4.1			Hypothetical protein
PA2007	*maiA*	15		16				Maleylacetoacetate isomerase
PA2008	*fahA*	8.1		6.9				Fumarylacetoacetase
PA2009	*hmgA*	7.8		9.5				Homogentisate 1,2-dioxygenase
PA2540		28						Conserved hypothetical protein
PA3038				2.3		2.8		Probable porin
PA3234				3.6		4.0		Probable sodium:solute symporter
PA3235				2.9	2.9	5.4		Conserved hypothetical protein
PA3205			6.7		2.7			Hypothetical protein

[a] These genes have been reported previously as being regulated by quorum sensing (either QS regulated or QS repressed) (43, 51). CoA, coenzyme A; MFS, major facilitator superfamily; FAD, flavin adenine dinucleotide.

TABLE 3. Summary of quorum-sensing-related genes that were down regulated in (i) isolate LES400 compared to strain PAO1, (ii) isolate LES431 compared to strain PAO1, and (iii) isolate LES431 compared to isolate LES400

ORF[a]	Gene name	LB LES400 ↓ vs PAO1	H_2O_2 LES400 ↓ vs PAO1	LB LES431 ↓ vs PAO1	H_2O_2 LES431 ↓ vs PAO1	LB LES431 ↓ vs 400	H_2O_2 LES431 ↓ vs 400	Product description
QS activated								
PA0026		4.3						Hypothetical protein
PA0105	*coxB*						6.6	Cytochrome *c* oxidase subunit II
PA0107							14	Conserved hypothetical protein
PA1317	*cyoA*		13					Cytochrome *o* ubiquinol oxidase subunit II
PA1319	*cyoC*		4.56					Cytochrome *o* ubiquinol oxidase subunit III
PA1404							4.8	Hypothetical protein
PA1431	*rsaL*	35						Regulatory protein RsaL
PA1432	*lasI*	12						Autoinducer synthesis protein LasI
PA2365							3.2	Conserved hypothetical protein
PA2445	*gcvP2*			3.8	2.9			Glycine cleavage system protein P2
PA2446	*gcvH2*			3.9	2.2			Glycine cleavage system protein H2
PA3181			9.7					2-Keto-3-deoxy-6-phosphogluconate aldolase
PA3182	*pgl*		2.6					6-Phosphogluconolactonase
PA3183	*zwf*		5.9			2.1		Glucose-6-phosphate 1-dehydrogenase
PA3188		74		26				Probable permease of ABC sugar transporter
PA3190		46	9.6	38	34			Probable binding protein component of ABC sugar transporter
PA3195	*gapA*				6.0			Glyceraldehyde 3-phosphate dehydrogenase
PA3369							4.0	Hypothetical protein
PA3418	*ldh*						6.4	Leucine dehydrogenase
PA3691							3.6	Hypothetical protein
PA3692							4.5	Probable outer membrane protein precursor
PA3923		5.9						Hypothetical protein
PA4131		7.2						Probable iron-sulfur protein
PA4133		4.1						Cytochrome *c* oxidase subunit (cbb3 type)
PA4311							5.7	Conserved hypothetical protein
PA4496		8.1	4.9	2.5	11			Probable binding protein component of ABC transporter
PA4498		14	34	3.1	31			Probable metallopeptidase
PA4876	*osmE*						8.0	Osmotically inducible lipoprotein OsmE
PA4880							3.3	Probable bacterioferritin
PA4916					12			Hypothetical protein
PA4917					5.0			Hypothetical protein
PA5027					9.5			Hypothetical protein
QS repressed								
PA0509	*nirN*		4.4		12			Probable *c*-type cytochrome
PA0510					40			Probable uroporphyrin-III c-methyltransferase
PA0512		8.4	15		17			Conserved hypothetical protein
PA1559						3.9		
PA2007	*maiA*						18	Maleylacetoacetate isomerase
PA2008	*fahA*						19	Fumarylacetoacetase
PA2009	*hmgA*						7.8	Homogentisate 1,2-dioxygenase
PA2259	*ptxS*		2.8		5.1			Transcriptional regulator PtxS
PA2260					4.3			Hypothetical protein
PA2261					11			Probable 2-ketogluconate kinase
PA2540						4.8		Conserved hypothetical protein
PA3174					3.2			Probable transcriptional regulator
PA3205							2.6	Hypothetical protein
PA3364	*amiC*	24		4.6	12			Aliphatic amidase expression-regulating protein
PA3365		6.6	5.7	5.1	10			Probable chaperone
PA3391	*nosR*		13		38			Regulatory protein NosR
PA3392	*nosZ*	62	23	42	56			Nitrous oxide reductase precursor
PA3393	*nosD*	5.9		4.6	7.3			NosD protein
PA3394	*nosF*				8.1			NosF protein
PA3575					2.7			Hypothetical protein
PA3662		4.2	7.7	12	20			Hypothetical protein
PA3790	*oprC*	9.7	37	7.4				Putative copper transport outer membrane porin OprC precursor
PA3872	*narI*	2.8						Respiratory nitrate reductase gamma chain
PA3877	*narK1*				9.1			Nitrite extrusion protein 1
PA3913		10		4.7	9.7			Probable protease
PA4442	*cysN*				2.3			ATP sulfurylase GTP-binding subunit/APS kinase
PA4587	*ccpR*	10	3.6	5.6	44		12	Cytochrome *c*551 peroxidase precursor
PA4770	*lldP*				2.9			L-lactate permease
PA4918		3.2	4.5	35	43	8.3		Hypothetical protein

[a] These genes have been reported previously as being regulated by quorum sensing (either QS activated or QS repressed) (43, 51).

TABLE 4. Summary of expression variations for genes associated with antimicrobial susceptibility[a]

ORF	Gene name	LB LES400 ↑ vs PAO1	H_2O_2 LES400 ↑ vs PAO1	LB LES400 ↓ vs PAO1	LB LES431 ↑ vs PAO1	H_2O_2 LES431 ↑ vs PAO1	LB LES431 ↓ vs PAO1	H_2O_2 LES431 ↓vs PAO1	LB LES431 ↑ vs 400	H_2O_2 LES431 ↑ vs 400	LB LES431 ↓ vs 400	H_2O_2 LES431 ↓ vs 400
PA0424	*mexR*	18	9.0		7.2	10						
PA0425	*mexA*	3.4				2.6						
PA0426	*mexB*	2.4										
PA0427	*oprM*	3.9									2.9	
PA0958	*oprD*			16			5.0	12				
PA2018	*mexY*	7.9				11						
PA2019	*mexX*	8.0	17		4.1	33						
PA2020	*mexZ*	3.2										
PA2493	*mexE*	16	66		18	295				5.1		
PA2494	*mexF*					57						
PA2495	*oprN*					6.0						
PA4110	*ampC*	12	46		137	178			11	4.0		
PA4205	*mexG*				45	8.7			5.9			
PA4206	*mexH*				11	6.7			9.2			
PA4207	*mexI*				15	8.4			6.2	4.9		
PA4208	*opmD*				4.3	8.0			5.5			
PA4599	*mexC*	28	10								32	43
PA4776	*pmrA*	6.7									6.6	
PA4777	*pmrB*	9.2										

[a] Arrows indicate isolates in which the gene is up-or down-regulated. LB or H_2O_2 indicate the two growth conditions used in this study. Values represent the average change in gene expression from replicate experiments.

associated genes of the cluster *pilMNOPQ* were down-regulated in isolate LES400 under both growth conditions but in isolate LES431 only under stress. The presence of this cluster has been associated with CF and may confer an early colonization or persistence advantage (18). We confirmed that following growth on Luria agar both LES isolates, unlike strain PAO1, lacked any visible flagella or pili, were nonmotile, and lacked twitching activity (data not shown).

Under oxidative stress, a large cluster of ribosomal proteins (PA4237 to PA4274) was down-regulated in isolate LES400 when compared to either strain PAO1 or isolate LES431. Genes associated with ribosomal biogenesis are known to be down-regulated following exposure to hydrogen peroxide (34). Similarly, several general secretory pathway genes (*secA*, *secB*, *secE*, and *secY*) were down-regulated in isolate LES400 in comparison to strain PAO1 or isolate LES431. Of the 13 *nuo* genes mostly clustered at PA2638 to PA2649 and encoding NADH dehydrogenase complex I, 10 were down-regulated in isolate LES400 and 5 were down-regulated in isolate LES431 when compared to strain PAO1. *nuoAL* genes were down-regulated in isolate LES400 compared to isolate LES431. F_1F_0 ATP synthase genes (PA5554 to PA5560) were also down-regulated in isolate LES400 compared to either strain PAO1 or isolate LES431. These data indicate a somewhat enhanced oxidative stress response, especially in isolate LES400, compared to strain PAO1.

Under stress, there was modest up-regulation (2.2 to 4.9 fold) of genes associated with DNA repair (*recA*, *lexA*, *recN*; PA0670 to PA0671) and of *sodB* (superoxide dismutase; 13 fold) in isolate LES431 compared to strain PAO1. *recA* was also up-regulated in isolate LES400.

Up-regulation of genes involved in antimicrobial susceptibility in LES isolates. We reported previously that antibiotic susceptibility profiles of LES isolates sharing PFGE genotypes can vary widely (35). The sensitivities of both LES isolates used in the microarray analysis and strain PAO1 to a number of antimicrobial agents are detailed in Table 5. Both LES isolates were less sensitive than strain PAO1 to β-lactams, aminoglycosides, and quinolones but with some notable variations between isolates LES400 and LES431. In particular, isolate LES431 was more resistant to the β-lactams piperacillin (in combination with the β-lactamase inhibitor tazocin) and imipenem (Table 5). Changes in gene expression that are likely to contribute to these variations in antimicrobial susceptibility are shown in Table 4 (12, 13, 19, 33, 50, 58).

We found a number of mutations in loci associated with antimicrobial susceptibility (summarized in Table 6). Expression of *ampC* was up-regulated in both LES isolates compared to strain PAO1, but the level of expression in isolate LES431 was >10-fold higher (Table 4). To resolve the variation in *ampC* expression between the two LES isolates, we sequenced the *ampR* genes and *ampR-ampC* intergenic regions, including

TABLE 5. Antimicrobial agent susceptibility profiles of the isolates

Antimicrobial agent	MIC value (μg/ml)		
	LES400	LES431	PAO1
Piperacillin/tazocin	8	64	1
Aztreonam	48	32	0.75
Ceftazidime	16	16	0.5
Imipenem	0.2	4	0.38
Meropenem	6	6	0.25
Imipenem (+ EDTA)	0.2	1	<1
Gentamicin	4	2	0.5
Amikacin	16	12	1
Tobramycin	1	0.75	0.19
Colistin	0.19	0.19	0.19
Ofloxacin	4	3	0.5
Ciprofloxacin	0.5	1	0.032
Cotrimoxazole	2	0.25	0.15

TABLE 6. Summary of mutations in regions associated with antimicrobial susceptibility and QS

Locus	LES isolate(s)[a]	Mutation(s)	Comment(s)	Relevant reference(s)
Antimicrobial Susceptibility				
mexR	LES400 and LES431	R_{83}→C	Mutation not reported previously	13, 24, 32, 57
mexA-R intergenic	LES400 and LES431	Ribosome-binding site for *mexA* AGGA → AGGG	Previous report of mutation in the ribosome-binding site leading to down-regulation (AGGA → CGGA)	13
ampR	LES400 and LES431	D_{135}→G;	Previous report of D_{135}→N mutation in strain with high levels of β-lactamase activity; disruption of AmpR translation	2
ampR-C intergenic	LES400 only	ATG→TTG (start codon) −71 with respect to the start codon of *ampG*$_5$ C→T	Effect of mutation is unknown	
ampC	LES400 only	ATG→ATT (start codon)	Disruption of AmpC translation	
ampD	LES400 and LES431	G_{148}→A and S_{175}→L	Same substitutions observed previously in strains with high AmpC activity but discounted as cause of up-regulation	2, 19
PA4523-ampD intergenic	LES400 and LES431	76 bp upstream of the *ampD* start codon; A→C	Effect of mutation is unknown	
mexZ	LES400 and LES431	Q_{164}→termination (CAA→TAA)	Similar though not identical truncations associated with aminoglycoside resistance among CF isolates	49
QS related				
rhlR-rhlI intergenic	LES400 and LES431	30 bp upstream of the *rhlI* start codon, TTTTTTTTCTC → TTTTTTT-CTC	Effect of mutation is unknown	
rhlB	LES400 and LES431	Downstream of P3 (one *rhlR* transcription start site), AGGGAGGGGGATGCTC → AGGGAGGGGGATGCGC	Effect of mutation is unknown	
lasR	LES400 only	Repetition of GGTGCTC leading to divergence from G_{123} onward	Loss of the HTH DNA-binding LuxR motif	
lasR	LES430 only	Deletion of GTGGATGCTC leading to divergence from position W_{152} onward	Loss of the HTH DNA-binding LuxR motif	
lasR	LESB44 only	Insertion of GAAG leading to divergence from position I_{35} onward	Loss of most of LasR	
rsaL-lasI intergenic	LES400 and LES431	In between the *lux*-box-like sequence NNCT-$(N)_{12}$-AGNN and the *lasI* start codon, located 6 bp away from the *lux* box-like sequence	Effect of mutation is unknown	
PA2587 and *gacA* intergenic	LES400 and LES431	6 bp different from PAO1; nearest mutation was 160 bp upstream from the *gacA* start codon	Effect of mutation is unknown	

[a] LES isolates refers to LES400 and LES431 only, with the exception of the mutations found in the *lasR* gene of isolates LES430 and LESB44.

the start of *ampC*. The predicted AmpR protein sequence for the LES isolates differed in one internal position compared to PAO1 (D_{135}→G). A similar mutation (D_{135}→N) has been reported previously in a strain exhibiting high levels of β-lactamase activity (2). However, the major difference was the replacement of the *ampR* ATG start codon with the sequence TTG, a mutation likely to render the sequence untranslatable. Since AmpR is reported to be a positive regulator for *ampC* (24), this observation was counter to what might be expected. It has been reported that mutations in AmpR can lead to enhanced expression of chromosomal β-lactamase in *P. aeruginosa* (2), but isolate LES431 in particular is a strain with high constitutive expression of *ampC* that lacks a functional AmpR, which suggests that activation by AmpR is not a prerequisite for high levels of AmpC.

Intriguingly, there were two further mutations in isolate LES400 when compared to both isolate LES431 and strain PAO1, one in the *ampR-ampC* intergenic region and another leading to mutation of the *ampC* start codon (Table 6). Thus, although expression of *ampC* transcription may be up-regulated in isolate LES400 compared to that in strain PAO1, it is likely that the AmpC protein cannot be translated.

Inactivation of the *ampD* gene has been associated with increased levels of AmpC β-lactamase in *P. aeruginosa* (2, 19). We amplified and sequenced the *ampD* genes and the PA4523-*ampD* intergenic regions of isolates LES400 and LES431 and found them to be identical. The AmpD predicted protein sequence differed in two positions when compared to strain PAO1 (Table 6). Identical substitutions have been observed in more than one other strain associated with high basal levels of

AmpC activity (19). However, the same mutations were also present in strains with low basal levels and inducible AmpC, suggesting that such mutations cannot explain the high basal levels observed in strain LES431 (19). In addition, there was a single nucleotide variation between LES strains and strain PAO1 in the PA4523-*ampD* intergenic region (Table 6).

In support of the gene expression data, using the chromogenic cephalosporin nitrocefin we were able to detect strong β-lactamase activity in isolate LES431. In contrast, β-lactamase activity in isolate LES400 was barely detectable (unpublished data). It has been reported previously that imipenem is not a substrate for the MexAB-OprM or MexXY efflux pumps (27). Since imipenem resistance changes in the presence of a metallo-β-lactamase inhibitor (EDTA) (Table 5), it seems more likely that imipenem resistance and the variation in MICs between the LES isolates are largely due to the production of β-lactamase from the *ampC* gene. MICs for ceftazidime were the same for LES400 and LES431, suggesting that although ceftazidime resistance has been used to measure of *ampC* activity (2), other factors can contribute. It has been reported previously that some *P. aeruginosa* isolates can be more susceptible to β-lactam antibiotics such as ceftazidime despite overproduction of both AmpC and MexAB-OprM, although the mechanisms behind this are unclear (12).

Overproduction of MexAB-OprM can significantly enhance resistance by *P. aeruginosa* to a range of drugs (17, 22, 32, 57, 58). A number of different mutations associated with overexpression of the MexAB-OprM efflux pump have been reported. *nalB* mutants arise from nucelotide sequence variations in the adjacent *mexR* repressor gene (37, 39, 46, 58). Cao et al. (4) have reported that *nalC* mutants carry mutations in the gene *PA3721* (renamed *nalC*), whose product appears to repress the genes *PA3720* and *PA3719*. Up-regulation of these genes may contribute to the *nalC* mutant phenotype. However, we did not observe any alterations in expression between LES strains and PAO1 for the *PA3719* to *PA3721* genes.

We sequenced the *mexR* gene and the *mexA-mexR* intergenic region of the two LES isolates. Both LES400 and LES431 carry a single amino acid change in the predicted MexR protein when compared to PAO1. Isolates LES400 and LES431 also carry a single nucleotide change in the intergenic region between *mexR* and *mexA* in the putative ribosome-binding site for *mexA*. In a previous study, Hocquet et al. (12) complemented various *mexR* mutations but concluded that this efflux pump contributed only marginally to β-lactam and fluoroquinilone resistance. However, it has been suggested that the MexAB-OprM pump plays a role in *P. aeruginosa* invasiveness and may be involved in the delivery of virulence factors to host cells (11). Hocquet et al. (12) have speculated that overexpression of this efflux system may contribute to the success of an epidemic clone by playing an important role in virulence rather than antibiotic resistance. It has been reported that a QS autoinducer can enhance *mexAB-oprM* without MexR-mediated regulation (41). This activity is repressed by MexT (*PA2492*). However, we observed no variation in expression of *mexT*.

In a study of *P. aeruginosa* clinical isolates, carbapenem resistance was linked with the loss of or decreased levels of OprD (33). In isolates LES400 and LES431, expression of *oprD* was down-regulated (Table 4) compared to PAO1. MICs of meropenem were reported to be two to four times higher for isolates expressing MexAB-OprM in a background of low OprD levels (33). It seems likely that these variations in gene expression also contributed to the 24-fold increase in MICs for meropenem in the LES strains compared to PAO1.

The MexXY system enables *P. aeruginosa* to become resistant to aminoglycosides, tetracyclines, and macrolides (13, 26) and has been specifically implicated in the emergence of resistance to aminoglycosides in CF isolates (50). There was evidence of up-regulation of the MexXY system in LES strains compared to PAO1 under the conditions used for growth in this study (Table 4). Expression of this system is normally inducible by aminoglycosides under the control of the MexZ repressor. It has been demonstrated that mutations in *mexZ* are associated with overproduction of the MexXY efflux system and increased resistance to aminoglycosides (50), and CF isolates have been shown to overproduce this system constitutively (53). Sequencing of the *mexZ* genes and *mexZ-mexX* intergenic regions of isolates LES400 and LES431 revealed that the gene was identical in the two LES isolates, differing by five nucleotides from the PAO1 sequence. Although four of these five mutations were synonymous nucleotide substitutions, the fifth introduced a stop codon leading to premature termination of the MexZ protein after 163 amino acids, compared to the length in strain PAO1 of 210 amino acid residues (Table 6). Similar although not identical truncations have been reported previously and implicated strongly in the development of stable aminoglycoside resistance among CF isolates of *P. aeruginosa* (50). The *mexZ-mexX* intergenic regions were identical in the three strains. These data suggest that the mutation in *mexZ* contributes to the up-regulation of the MexXY system and the greater resistance to aminoglycosides in the LES isolates.

Clinical strains that simultaneously overproduce the MexAB-OprM and MexXY efflux pumps have been reported previously (25). The LES isolates overexpress both efflux pumps and the AmpC β-lactamase. It has been suggested that simultaneous expression of two or three Mex pumps (MexAB-OprM, MexCD-OprJ, and MexEF-OprN) has an additive effect on the MICs of relevant antimicrobial agents (21). Clearly, the LES produces a considerable armory with which to defend itself from antimicrobial agents. Yet as well as some isolates displaying this prowess to the full, LES populations include isolates, such as LES400, with mutations removing some of these weapons.

Premature expression of QS-regulated genes in some LES isolates. In *P. aeruginosa*, numerous genes, including many known virulence genes, are regulated by the two *lux*-like QS systems *rhl* and *las* (44, 52), and these genes were up-regulated in isolate LES431 (Tables 1 to 3). Assays for elastase, LasA, and pyocyanin confirmed the high-level expression of these QS-regulated activities in isolate LES431 and indicated premature induction of the QS system (Fig. 2). Interestingly, we detected premature pyocyanin production in an isolate from the infected non-CF father of a CF patient (LES431) and in isolates from the non-CF mother (LES417) and CF patient (LES416) from the same infection episode (Fig. 2c) (29).

To identify mutations in important QS regulatory genes that could account for the observed variations in transcription of QS-related genes, we sequenced the *lasR-rsaL-lasI*, *rhlIR*, *vfr*, *gacA*, and *qscR* regions of the LES400 and LES431 isolates.

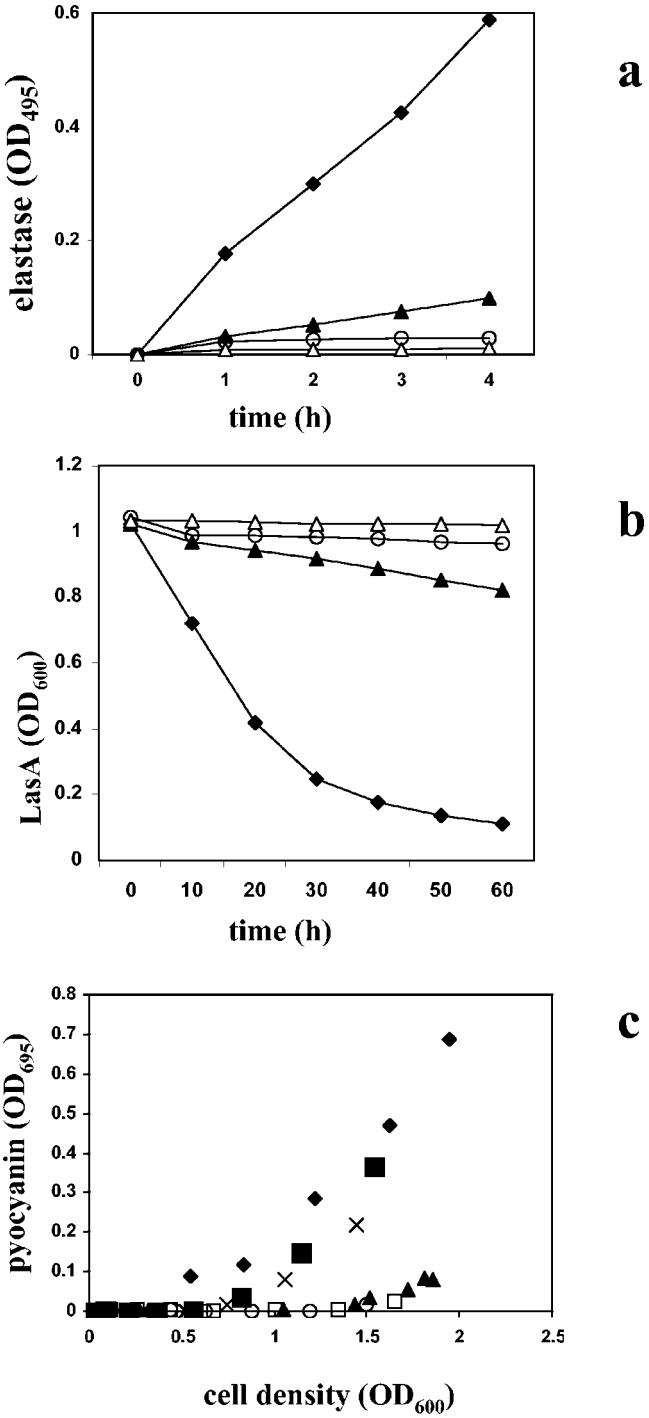

FIG. 2. Expression of QS-regulated phenotypes. Production of elastase (a), LasA (b), and pyocyanin (c) in *P. aeruginosa* PAO1 (▲), LES400 (○), LES431 (◆), LES416 (■), LES417 (x), and LES430 (□). △, LB control.

The mutations found are summarized in Table 6. Significantly, in isolate LES400 alone, there was a repetition of a heptanucleotide sequence (GGTGCTC) within the coding region of LasR, leading to divergence from the normal LasR sequence from position G_{123} onwards, resulting in the complete loss of the HTH DNA-binding LuxR motif. Interestingly, a second isolate from the non-CF father in the infection episode (LES430) did not share the QS phenotype of isolate LES431 (Fig. 2c). In isolate LES430, there was a 10-bp deletion within the LasR-coding sequence, again leading to loss of the DNA-binding domain (Table 6). We also identified an LES isolate from the CF patient (LESB44) that lacked the unusual QS phenotype and carried a different mutation in *lasR* (Table 6). Isolates LES430 and LESB44 both had pyocyanin and elastase activities that were similar to those of isolate LES400. The only other difference between LES isolates and PAO1 in the *lasR-rsaL-lasI* region was a mutation close to the *lux*-box-like element thought to control both *rsaL* and *lasI* expression (Table 6) (54). This mutation was also carried by isolates LES416, LES417, and LES430.

A number of previous studies have identified genes with a role in the regulation of the QS system, including *qscR* (8), *gacA* (20), *vfr* (30), *rpoS* (43, 55), and *rpoN* (10). Neither nucleotide sequencing nor analysis of gene expression data yielded any compelling evidence for the involvement of these genes in the QS phenotype of isolate LES431. We did identify in both LES isolates a 6-bp difference in the intergenic region between *PA2587* and *gacA*. *gacA* is up-regulated in isolate LES400 compared to strain PAO1 (2.4 fold) following growth in LB and in isolate LES431 compared to strain PAO1 following oxidative stress (3.2 fold), but these variations cannot account for the QS phenotype of isolate LES431.

Although the predicted RhlI and RhlR protein sequences for the LES isolates were identical to those of strain PAO1, there was a 1-bp deletion in both LES400 and LES431 30 bp upstream of the *rhlI* start codon (Table 6). The intergenic region between *rhlB* and *rhlR* was identical in all three strains. However, it has been reported that the *P. aeruginosa* PAO1 *rhlR* gene has four transcription start sites (P1 to P4), two of which are within the *rhlB* coding region (30). We observed a 1-bp difference between the LES strains and strain PAO1 near the end of the *rhlB* coding region (Table 6). This mutation is also present in the sequence reported by Medina et al. (30) as PAO1, even though it differs from the strain PAO1 genome sequence. Since during growth in LB medium, *rhlR* is expressed from promoter P2, and promoter P3 is thought to be σ^{54} dependent (30), this mutation is unlikely to play a role in the observed differences in transcription during growth in LB.

A recent report highlighted the existence of QS-deficient clinical isolates of *P. aeruginosa* (42), concluding that QS-deficient strains are capable of causing infections. In the study by Schaber et al. (42), PCR assays suggested that two of five QS-deficient strains lacked *lasR* and *rhlR* genes. In our study, we show that the same strain (LES) can exhibit phenotypes from premature and excessive production of the QS system to QS deficiency, the latter being due to naturally occurring frameshift mutations in the *lasR* gene. Although we do not unequivocally identify mutations that account for the up-regulation in LES431 and other isolates, the mutation upstream of *lasI* may play a role in this phenotype.

Evidence from an infection model of the greater virulence of isolate LES431. Chugani et al. (8) demonstrated the increased virulence of a *qscR* mutant of strain PAO1 compared to the wild type in a *D. melanogaster* infection assay. We compared the virulence of isolate LES431, which shares a similar QS phenotype to *qscR* mutants, with isolate LES400, which carries

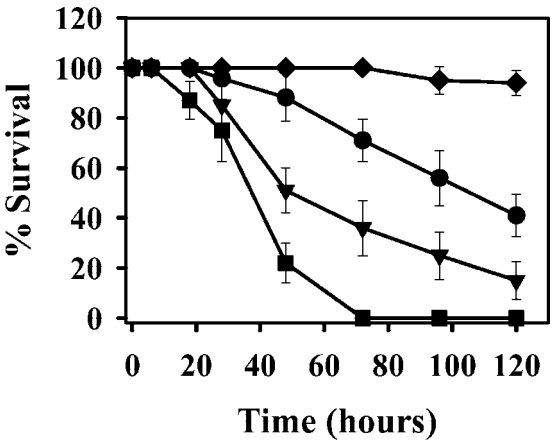

FIG. 3. Virulence of *P. aeruginosa* in fruit flies. Deaths of flies over time when fed *P. aeruginosa* PAO1 (●), LES400 (▼), LES431 (■), or buffer (◆) are shown.

a *lasR* mutation, and strain PAO1. Isolate LES431 clearly gave the phenotype described previously as hypervirulence (Fig. 3). It is interesting that isolate LES400 was more virulent than strain PAO1 in this infection model, which suggests that even LES isolates lacking the QS-regulated virulence genes cannot be considered avirulent.

The LES may represent a transmissible, hypervirulent clone. In common with chronic CF infections by *P. aeruginosa*, the LES exhibits considerable phenotypic diversity in properties such as antimicrobial sensitivity and colony morphology (34). What distinguishes the LES from most other *P. aeruginosa* strains is its inherent ability to transmit between CF patients and, in the case of isolate LES431, to cause infections in non-CF parents of CF patients. Although our study does not definitively identify the factors responsible for these additional abilities, we demonstrate the flexibility and adaptability of this strain. In particular, isolate LES431, which because of its history might be considered a highly virulent variant of the clone, has high levels of β-lactamase activity coupled with up-regulation of QS-regulated virulence genes. In contrast, isolate LES400, associated with a chronic CF infection, carries a specific LasR mutation leading to loss of QS activity and has also lost AmpC β-lactamase activity, due to a point mutation in the *ampC* start codon. It may be that either during the course of chronic CF infections or during laboratory culture, isolate LES400 lost the requirement for these and other properties. However, both LES isolates retained resistance to several other antimicrobials, some of which are due to shared mutations.

von Götz et al. (51) have demonstrated previously that CF strains can evolve into variants expressing higher levels of virulence genes, contradicting the general assumption that selection during CF will have a tendency to reduce such expression. It is possible that the QS phenotype displayed by isolate LES431 may have contributed to its aggressive abilities. This raises the possibility that isolate LES431 represents a novel variant of LES that has evolved from a clone already known to be a successful colonizer of CF patients. However, we have observed that the QS phenotype of isolate LES431 is shared by not only isolates from other CF patients, but also the oldest known LES isolate (from 1988). This suggests that rather than evolving from a successful CF clone, the novel QS phenotype was an intrinsic characteristic of the LES that has been lost subsequently by some isolates, due to mutations such as those we have observed with the *lasR* gene. It may be that the hypervirulence phenotype is a rare, short-lived phenomenon creating an opportunity for transmission and infection beyond what is normal but placing a burden on the bacterium, leading to the selection for subsequent mutations and then loss of the QS system. In the context of CF infections, the emergence of a hypervirulent, transmissible strain constitutes a potential concern for infection control, which at present is aimed solely at CF patients and not at non-CF parents and health workers.

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