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Virulence Properties of *Pseudomonas aeruginosa* Lacking the Extreme-Stress Sigma Factor AlgU (σ^E)

H. YU, J. C. BOUCHER, N. S. HIBLER, AND V. DERETIC*

Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7758

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A discerning feature of *Pseudomonas aeruginosa* strains causing chronic endobronchial infections in cystic fibrosis is their conversion into the mucoid, exopolysaccharide alginate-overproducing phenotype. This morphologically prominent change is caused by mutations which upregulate AlgU (σ^E), a novel extreme-stress sigma factor with functional equivalents in gram-negative organisms. In this work, we investigated the role of *algU* in *P. aeruginosa* sensitivity to reactive oxygen intermediates, killing by phagocytic cells, and systemic virulence of this bacterium. Inactivation of *algU* in *P. aeruginosa* PAO1 increased its susceptibility to killing by chemically or enzymatically generated halogenated reactive oxygen intermediates and reduced its survival in bactericidal assays with J774 murine macrophages and human neutrophils. Surprisingly, inactivation of *algU* caused increased systemic virulence of *P. aeruginosa* in mouse models of acute infection. The increased lethality of the *algU*-deficient strain was also observed in the endotoxin-resistant C3H/HeJ mice. Only minor differences between *algU*$^+$ and *algU* mutant cells in their sensitivity to human serum were observed, and no differences in their lipopolysaccharide profiles were detected. Intriguingly, while inactivation of *algU* downregulated five polypeptides it also upregulated the expression of seven polypeptides as determined by two-dimensional gel analyses, suggesting that *algU* plays both a positive and a negative role in gene expression in *P. aeruginosa*. While the observation that *algU* inactivation increases systemic virulence in *P. aeruginosa* requires further explanation, this phenomenon contrasts with the apparent selection for strains with upregulated AlgU during colonization of the cystic fibrosis lung and suggests opposing roles for this system in chronic and acute infections.

Pseudomonas aeruginosa causes serious and often fatal opportunistic infections ranging from systemic acute disease in burned and neutropenic patients (3) to chronic infections of the respiratory tract in cystic fibrosis-infected patients (CF patients) (14). Chronic colonization with *P. aeruginosa* in CF is frequently accompanied by a series of unusual phenotypic changes (8, 18, 29, 30, 51), with the most prominent morphological feature being conversion to mucoidy caused by the overproduction of the exopolysaccharide alginate (6, 10, 38, 55). Alginate plays a multifactorial role in the pathogenesis of the CF lung (38). Among performing other functions, it inhibits phagocytosis by neutrophils and macrophages (38, 51) and insulates *P. aeruginosa* from reactive oxygen intermediates and hypochlorite generated by the phagocytic cells of the host (25, 48–50).

In their seminal studies, Fyfe and Govan (13) reported that mutations in the *muc* loci, mapping in the late region of the *P. aeruginosa* chromosome, cause conversion to mucoidy. More recently, the molecular characterization of this locus has led to the identification of five tightly linked genes, *algU mucABCD*, in this region (4, 32–34, 55). The *algU* gene (also known as *algT* [9, 16]) encodes an alternative σ factor which is a founding member of a new class of stress-responsive alternative sigma factors (7, 28) and is functionally interchangeable (56) with the extreme-stress sigma factor σ^E (RpoE) from enteric bacteria (11, 40, 45). For members of the family *Enterobacteriaceae*, σ^E has been implicated in the activation of heat shock response and defense systems necessary for survival at extreme temperatures or during exposure to reactive oxygen intermediates (22, 27).

While very little is known about the possible role of AlgU (σ^E) homologs in the virulence of enteric bacteria, it has been established that AlgU is the principal regulator of mucoidy (7, 32, 55), a well-recognized pathogenic determinant expressed by *P. aeruginosa* in CF (24, 38). In the course of the establishment of chronic respiratory infections in CF, AlgU is upregulated because of the mutations in the downstream genes (4, 34), which most frequently occur in *mucA* (4, 34, 55), a gene encoding the AlgU cognate anti-sigma factor (47). The uncovering of the nature of genes and factors controlling conversion to mucoidy suggests that mucoidy may be only one manifestation of the activation of the alternative sigma factor AlgU. Since AlgU is a functional equivalent of the extreme-heat shock sigma factor σ^E (56), it appears possible that AlgU, in addition to regulating alginate production, may affect expression of additional stress response systems of possible relevance for pathogenesis. We have recently reported that *algU* mutants of *P. aeruginosa* show increased susceptibility to intracellularly generated superoxide (35, 56), thus extending the role of *algU* beyond that of the control of alginate production. In this study, we analyzed the susceptibility of *P. aeruginosa algU* mutants to reactive oxygen intermediates relevant for in vivo conditions, tested their survival during interactions with macrophages and human neutrophils, and examined the effects of *algU* inactivation on the systemic virulence of *P. aeruginosa* in mouse models of acute infection.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *P. aeruginosa* was grown on *Pseudomonas* isolation agar (Difco) or in Luria-Bertani (LB) broth supplemented with 150 μg of gentamicin per ml or 300 μg of carbenicillin per ml when required. PAO6862, a derivative of PAO1 with chromosomally inactivated *algD*,

* Corresponding author. Present address: Department of Microbiology and Immunology, 5641 Medical Science Building II, University of Michigan Medical School, Ann Arbor, MI 48109-0620. Phone: (313) 763-1580. Fax: (313) 764-3562. Electronic mail address: Deretic @umich.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant properties[a]	Reference or source
P. aeruginosa		
PAO1	*algU*$^+$ *algD*$^+$ *algW*$^+$ *mucD*$^+$ Alg^{-wt}	B. Holloway
PAO6852	PAO1 *algU*::Tc^r	32
PAO6854	PAO1 *algW*::Tc^r	4
PAO6860	PAO1 Alg^{+m} *mucD*::Gm^r	4
PAO6862	PAO1 *algD*::Gm^r	This work
Plasmids		
pHYAG	pVDtac39 *rpoE*$^+$ *mclA*::Gm^r	56
pHYDGX	pCMobB *algD*::Gm^r *mob* Ap^r (Cb^r)	This work

[a] Alg^{-wt}, nonmucoid wild type; Alg^{+m}, medium-dependent mucoid phenotype.

was generated as follows: pHYDX (56), a suicide plasmid which contains *xylE* on a 2.2-kb *Xho*I fragment inserted in the *Xho*I site of *algD*, was modified by replacing the *xylE* insert with the gentamicin resistance cassette (1.8-kb *Xho*I fragment) from pGmXho (4). Gene replacements were verified by Southern blotting. For the determination of 50% lethal doses (LD_{50}s), *P. aeruginosa* was grown in LB broth at 37°C to an optical density at 600 nm (OD_{600}) of 0.4. The culture was pelleted by centrifugation at 3,000 × *g* and washed twice in cold 1% Proteose Peptone (Difco) in 10 mM phosphate-buffered saline (PBS) (pH 7.4). The final cell pellet was resuspended in PBS–32.5% glycerol, and infectious doses were aliquoted and rapidly frozen at −80°C. Viable-cell counts (CFU) in each infectious dose were determined at the time of infection. Bacterial viability in all bactericidal assays was determined by plating on LB.

Hypochlorite killing and disk sensitivity assays. Sensitivity of *P. aeruginosa* in suspension to NaOCl was tested according to the method of Learn et al. (25). *P. aeruginosa* was grown in 50 ml of LB broth until an OD_{600} of 0.4 was reached, was centrifuged at 3,000 × *g*, and was washed in 10 mM PBS (pH 7.4), and then the cells were resuspended in PBS–10 mM glucose (PBS-G) to an OD_{600} of 0.25 and allowed to rest for 30 min at 37°C. One milliliter of this suspension (approximately 2×10^8 CFU/ml) was added to 9 ml of prewarmed NaOCl solution (final concentration, 25 μM) in PBS-G and incubated at 37°C. Surviving bacteria were quantitated by plating after dilution in PBS. Disk sensitivity assays were carried out as previously described (4, 35, 56).

Myeloperoxidase-H_2O_2-chloride antimicrobial system. Myeloperoxidase-mediated oxidative killing of *P. aeruginosa* was carried out according to published procedures (41). The complete myeloperoxidase microbicidal system contained 0.3 U of myeloperoxidase (5 U/ml; EC 1.11.1.7; [from human leukocytes]; Sigma), 0.4 U of glucose oxidase (1,130 U/ml; type V-S [from *Aspergillus niger*]; Sigma), 0.1 M NaCl, 0.04 M sodium acetate (pH 5.0), 0.01 M glucose, 0.01 M Na_2SO_4, and 0.05 mg of gelatin in a total reaction volume of 10 ml. A total of 10^8 CFU (1 ml) from a mid-log-phase *P. aeruginosa* culture was added to 9 ml containing the nonenzymatic components of the system. The bactericidal reaction was initiated by adding myeloperoxidase and glucose oxidase. In control samples, myeloperoxidase was omitted.

Neutrophils and macrophage-mediated bactericidal assays. The procedures for the isolation of human neutrophils and the bactericidal assays were based on previously described methods (36). Heparinized blood was mixed by inversion with an equal volume of prewarmed 3% dextran (T500; Pharmacia) in 0.85% NaCl, and erythrocytes were sedimented by gravity for 18 min at room temperature. Leukocytes were collected by centrifugation at 500 × *g* for 10 min at 4°C, pellets were resuspended in 40 ml of cold 0.85% saline, and a 10 ml Ficoll-Hypaque cushion was layered beneath the cell suspension. The 9.97% (wt/vol) Hypaque (sodium diatrizoate; Winthrop, New York, N.Y.) and 6.35% (wt/vol) Ficoll (T400; Pharmacia) had a density of 1.08. After centrifugation at 250 × *g* for 40 min at 20°C, neutrophil pellets were resuspended in 10 ml of 0.2% NaCl and incubated for 20 s to hypotonically lyse the remaining erythrocytes. Immediately following the lysis of erythrocytes, 10 ml of 1.5% NaCl was added to balance the tonicity. Neutrophils were pelleted by centrifugation at 500 × *g* for 6 min at 4°C and resuspended in 10 ml of Hanks' buffered saline solution (pH 7.4). Neutrophils in Hanks' buffered saline solution (3×10^7 cells) were incubated with 3×10^8 CFU of *P. aeruginosa* in the presence of 10% heat-inactivated autologous human serum in a total volume of 10 ml. A volume of 1 ml was sampled for each time point by centrifugation at 900 rpm (Sorvall RT 6000 D) for 5 min and resuspending the pellet in an equal volume of cold sterile water to lyse the neutrophils before bacterial viability determination.

Macrophage-mediated killing of *P. aeruginosa* was carried out as follows. The mouse macrophage cell line J774 (ATCC TIB-67) was grown in Dulbecco's modified Eagle's medium (low glucose; Cellgro) supplemented with 5 mM L-glutamine and 5% fetal bovine serum (HyClone) in a 5% CO_2 atmosphere at 37°C. Mid-log-phase *P. aeruginosa* grown in LB broth at 37°C was collected by centrifugation at 4,000 rpm and resuspended to an OD_{600} of 0.4 in PBS. Macrophage cells (5×10^7) were incubated in Dulbecco's modified Eagle's medium–5% fetal bovine serum with 5×10^7 CFU of *P. aeruginosa* for 30 min at 37°C. In order to eliminate extracellular *P. aeruginosa*, three washes by centrifugation at 900 rpm for 5 min at room temperature in Hanks' buffered saline solution (Cellgro) were performed. After the final wash, macrophages were allowed to adhere to tissue culture flasks in Dulbecco's modified Eagle's medium supplemented with 400 μg of gentamicin per ml (42). Macrophages were harvested and resuspended in equal volumes of cold sterile water, and lysis was completed by vortexing the macrophages three times for 1 min and incubating the macrophages on ice for 15 min before bacterial viability was assessed by plating.

Serum sensitivity assay. The sensitivity of *P. aeruginosa* to human serum was tested by following published methods (36). For the bactericidal assay, 10^7 CFU of *P. aeruginosa* was incubated in 10% fresh serum in Hank's buffered saline solution (pH 7.4) and viability counts were determined by plating. For control experiments, sera were heated at 56°C for 30 min to inactivate the complement.

Neutropenic- and normal-mouse model of fatal *P. aeruginosa* septicemia. For the neutropenic-mouse model, 5- to 6-week-old C57BL/6 mice (average body weight, 23 g; Taconic) were rendered neutropenic by three intraperitoneal (i.p.) injections of 200 μg of cyclophosphamide per g of body weight every other day as previously described (5). Two days following the final dose of cyclophosphamide, the mice were challenged by i.p. injection (20) of bacteria in 0.2 ml of PBS. Mice in groups of five per dose were injected with inocula ranging from 10^1 to 10^3 CFU. For experiments with normal C57BL/6 or endotoxin-resistant C3H/HeJ mice, no cyclophosphamide was administered and bacterial doses ranged from 10^4 to 10^8 CFU. The same route of injection was used for all mice. The time required to cause mortality was recorded for determining the mean time to death.

Metabolic labeling of newly synthesized polypeptides and two-dimensional (2D) electrophoresis. De novo-synthesized proteins were analyzed according to a previously described procedure (46). Briefly, *P. aeruginosa* was grown in LB broth at 37°C until an OD_{600} of 0.4 was reached. A 200-μl aliquot of this culture was washed and resuspended in M9 glucose medium supplemented with 0.01% 18-amino-acid mix (without methionine and cysteine) and incubated at 30°C for 80 min. The culture was shifted from 30 to 50°C for a period of 5 min and labeled with [^{35}S]methionine and [^{35}S]cysteine (Expre^{35}S^{35}S protein label mix; 1,000 Ci/mmol; Du Pont NEN) at a final concentration of 33 μCi/ml for 5 min. Samples were rapidly chilled in a dry ice-ethanol bath and centrifuged at 5,000 rpm for 2 min at 4°C, and the cell pellets were washed in 0.85% NaCl. Cell extracts were equilibrated on ice for 2 h in lysis buffer containing 9.8 M urea, 2% Nonidet P-40, 2% ampholytes (pH 3 to 10), and 100 mM dithiothreitol. Samples were loaded on prefocused isoelectric focusing gels (pH 5 to 9) overlaid with the buffer containing 8 M urea, 5% Nonidet P-40, 1% ampholytes (pH 3 to 10), and 5% β-mercaptoethanol. The isoelectric focusing gels were run at 1,200 V overnight and then for an additional 45 min at 1,400 V. After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension, the proteins were electroblotted on an Immobilon-P membrane (Millipore) and analyzed by autoradiography.

Statistical analysis. Statistical analyses (analysis of variance and Fisher's protected least significant difference [PLSD]) were performed with SuperANOVA software (version 1.11; Abacus Concepts). The LD_{50}s were determined by Probit analysis with SPSS statistics software (PowerMac advanced version 6.1; SPSS Inc.) or by the Reed-Muench method (43, 54) as indicated.

RESULTS

Inactivation of *algU* in *P. aeruginosa* causes increased sensitivity to hypochlorite. During chronic lung infections in CF, *P. aeruginosa* is continuously exposed to the bactericidal products of polymorphonuclear leukocytes, which are present in high numbers in the lumen of CF lung tissue (2, 24, 38, 51). One of the halogenated reactive oxygen intermediates generated by human polymorphonuclear leukocytes with exquisite antipseudomonal activity is hypochlorous acid (25, 37). In order to test whether *algU* may play a role in *P. aeruginosa* susceptibility to hypochlorite, we tested *algU*$^+$ and *algU*::Tc^r *P. aeruginosa* for resistance to this compound. The results of disk inhibition zone assays with different oxidants are shown in Table 2, and the rate of killing with hypochlorite in bacterial suspension is displayed in Fig. 1A. Both analyses indicated that the *algU* mutant strain PAO6852 had increased sensitivity to hypochlorite relative to that of the parental *algU*$^+$ strain PAO1. While *algU*::Tc^r cells showed increased sensitivity to NaOCl and paraquat, there was no significant difference between PAO1 and PAO6852 in sensitivity to H_2O_2, in keeping with the previously reported observations (35) (Table 2).

TABLE 2. Sensitivities of *algU*::Tc^r *P. aeruginosa* to hypochlorite

Strain[a]	Genotype	Plasmid[b]	Mean diam (mm) of growth inhibition zone ± SE[c]		
			NaOCl (5%)	PQ (1.9%)	H_2O_2 (3%)
PAO1	*algU*$^+$		18.7 ± 0.4	12.5 ± 0.2	16.0 ± 0.3
PAO6852	*algU*::Tc^r		22.2 ± 0.6	18.5 ± 0.3	15.0 ± 0.3
PAO6852	*algU*::Tc^r	pHYAG	18.5 ± 0.4	10.8 ± 0.1	18.3 ± 0.2
PAO6862	*algD*::Gm^r		18.0 ± 0.5	12.6 ± 0.2	15.2 ± 0.2

[a] All strains were grown in LB broth at 37°C to an OD_{600} of 0.4.
[b] Plasmid pHYAG contains *Escherichia coli rpoE* which is interchangeable with *algU* and restores mucoidy in *algU*-null mutants of *P. aeruginosa* (56). *E. coli rpoE* was used instead of *algU* in complementation experiments, since plasmid-borne *algU* was unstable in *P. aeruginosa* in the absence of its negative regulators.
[c] Growth inhibition zones around 6-mm-diameter disks soaked with the indicated compounds (concentrations are given in parentheses) were measured after overnight incubation. The numbers represent the averages of nine measurements. The difference in sensitivity to NaOCl for PAO1 and PAO6852 (Fisher's PLSD test) was significant ($P < 0.0001$). PQ, paraquat.

Since alginate has been indicated as a scavenger of hypochlorite (25) and *algU* is functional in wild-type nonmucoid cells (35), it seemed possible that low levels of *algU*-dependent *algD* expression and the baseline alginate production in PAO1 could provide one explanation for the observed reduced resistance to hypochlorite in the *algU*-null mutant. To investigate this possibility, an isogenic *algD* mutant was generated by insertional inactivation of the *algD* gene on the chromosome of PAO1. When the resulting *algD*::Gm^r strain PAO6862 was tested, unlike PAO6852 (*algU*::Tc^r) this mutant did not display increased sensitivity to hypochlorite (Table 2). These results ruled out the possibility that *algU* inactivation resulted in sensitivity to hypochlorite due to the loss of basal alginate production.

Inactivation of *algU* causes increased sensitivity of *P. aeruginosa* to coupled human myeloperoxidase-glucose oxidase bactericidal system. We next tested whether *algU* mutant strains were more sensitive to enzymatically generated hypohalous ions, using a coupled human myeloperoxidase-glucose oxidase system. When PAO1 and PAO6852 were exposed to the complete myeloperoxidase-glucose oxidase system, PAO6852 (*algU*::Tc^r) was found to be somewhat more sensitive to killing by this acellular bactericidal system than was its parental strain PAO1 (Fig. 1B) (P [t test] for 30 min, 6.5×10^{-4}). When myeloperoxidase was omitted from the system, no killing of *P. aeruginosa* was observed (data not shown).

Inactivation of *algU* reduces *P. aeruginosa* survival in cellular bactericidal systems. Two assays were applied to compare the relative sensitivities of *algU*$^+$ and *algU*::Tc^r strains to bactericidal systems in phagocytes. The murine macrophage cell line J774 was used to examine the rate of macrophage-mediated killing of PAO1 (*algU*$^+$) and PAO6852 (*algU*::Tc^r) *P. aeruginosa*. Macrophages (5×10^7 cells) were infected at a multiplicity of infection of 1:1 and incubated for 1 h. The *algU* mutant strain PAO6852 was more susceptible to killing by macrophages than the parental *algU*$^+$ strain PAO1. After 1 h of incubation, PAO1 and PAO6852 showed 43.1% ± 2.9% and 18.3% ± 0.63% survival of the original input, respectively (P [t test], 1.7×10^{-4}). After 4 h of incubation, the number of surviving bacteria was less than 2% of the original input, and the differences at that time point were no longer significant. In a parallel set of experiments, the susceptibility of *algU*$^+$ and *algU* mutant cells to killing by human polymorphonuclear leukocytes was tested. Freshly prepared peripheral human blood neutrophils (3×10^7 cells) were incubated with PAO1 and PAO6852 (3×10^8 CFU) for 15 and 30 min. The results of these studies (Fig. 2) indicated that the *algU* mutant strain PAO6852 was more sensitive to the bactericidal action of human neutrophils than was the parental strain PAO1 (Fig. 2) (P [t test], 1.25×10^{-5} and 2.1×10^{-3} for 15 and 30 min, respectively).

Increased systemic virulence of *P. aeruginosa* with inactivated *algU* in neutropenic- and normal-C57BL/6 mouse models of fatal *Pseudomonas* septicemia. The experiments with *P. aeruginosa* survival in acellular and cellular bactericidal systems suggested that *algU* could play a role in *P. aeruginosa* virulence. In order to assess the effects of *algU* inactivation on systemic *P. aeruginosa* virulence, we determined LD_{50}s for *algU*$^+$ and *algU*::Tc^r strains in the neutropenic-mouse model of fatal *P. aeruginosa* sepsis (5, 20). C57BL/6 mice were rendered neutropenic by cyclophosphamide treatment (5) as described in Materials and Methods and challenged with *P. aeruginosa* i.p. (20). The three isogenic strains tested [PAO1 (wild type), PAO6852(*algU*::Tc^r), and PAO6854(*algW*::Tc^r) (used as a control)] did not show significant differences in LD_{50}s. The LD_{50}s ranged from 1.8×10^{-1} to 2.3×10^{-1} CFU as determined by the Reed-Muench method. However, the mean time to death caused by the challenge with the *algU*::Tc^r strain PAO6852 was significantly (P [analysis of variance] < 0.0001) shorter (31.1 ±

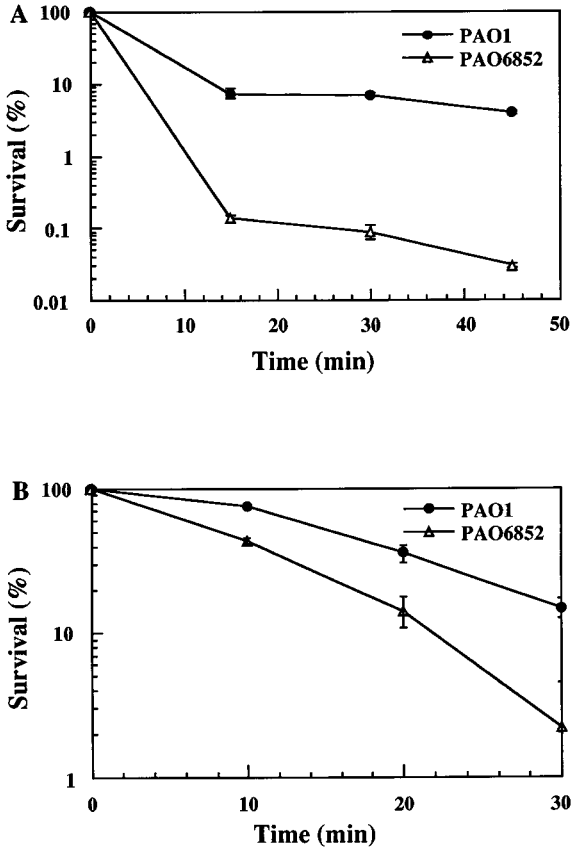

FIG. 1. Increased sensitivity of *algU* mutant *P. aeruginosa* to sodium hypochlorite (A) and to bactericidal action of the human myeloperoxidase-H_2O_2-Cl^- system (B). PAO1, wild-type *algU*$^+$; PAO6852, *algU*::Tc^r mutant of PAO1. PAO1 and PAO6852 (10^8 CFU each) were incubated in the presence of 25 μM NaOCl (A) or in a complete myeloperoxidase-glucose oxidase system (B). Survival is expressed as the fraction (percentage) of the initial cell input that survived the treatment. Bars, standard errors (each point is the average of three separate experiments). P values (t test) for the 15-min time point (A) and the 10-min time point (B) were 3.7×10^{-7} and 3.2×10^{-4}, respectively.

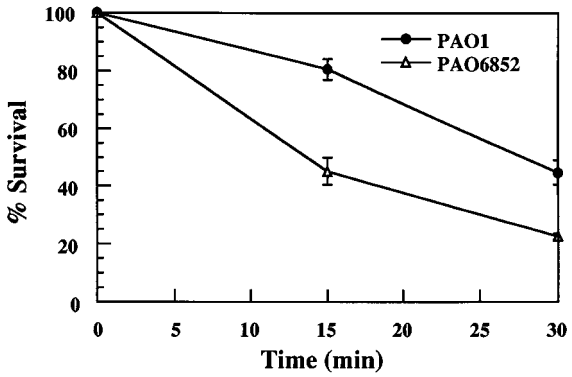

FIG. 2. Differential sensitivity of *algU*$^+$ (PAO1) and *algU*::Tc^r (PAO6852) strains to human neutrophils. Freshly prepared human neutrophils (3×10^7 cells) were incubated with isogenic *algU*$^+$ and *algU*::Tc^r cells of *P. aeruginosa* (3×10^8 CFU) in the presence of 10% heat-inactivated autologous human serum. Percent survival is defined in the legend to Fig. 1. *P* values (*t* test) for 15 and 30 min were 1.25×10^{-5} and 2.1×10^{-3}, respectively. Bars, standard errors (each point is the average of three independent experiments).

1.7 h [$N = 22$]) than that with the other strains tested (PAO1 and PAO6854) (55.9 ± 2.1 h [$N = 22$] and 52.3 ± 6.0 h [$N = 14$], respectively).

Since the cyclophosphamide-treated mice were neutropenic, it appeared possible that the lowered number of neutrophils might have rendered the system less responsive to differences in sensitivity observed with PAO1 and PAO6852 in bactericidal assays in vitro. In order to address this issue, we tested *P. aeruginosa* virulence in normal animals (without cyclophosphamide treatment). In order to ensure that LD_{50}s in this model can reflect increased susceptibility of *P. aeruginosa* to reactive oxygen species, we first tested another mutant of *P. aeruginosa* (strain PAO6860) with inactivated *mucD*. MucD is a homolog of HtrA, a factor controlled by σ^E in enteric bacteria (28, 40, 45), and is required for resistance to oxidative stress in *Salmonella typhimurium* (22); *P. aeruginosa mucD* mutants also show increased sensitivity to reactive oxygen intermediates (4). When PAO6860 was tested in the model of fatal *P. aeruginosa* septicemia in normal C57BL/6 mice, a level of virulence less than that with the wild-type PAO1 was observed (Table 3). The LD_{50} for PAO6860 (*mucD*::Gm^r) was approximately 20-fold higher than that for PAO1. We next tested whether inactivation of *algU* had an effect on the virulence of *P. aeruginosa* in the same model. The results of these experiments showed that *P. aeruginosa* PAO6852 had an LD_{50} 1 log lower than that of its parental strain PAO1 (Table 3). When subjected to Probit analysis (54), 95% confidence intervals for LD_{50}s were 7.5×10^5 to 2×10^6 CFU for PAO1 and 1.07×10^5 to 2.98×10^5 CFU for PAO6852, indicating that the differences observed were statistically significant. The finding that *algU* inactivation increased *P. aeruginosa* virulence was consistent with the observations in the neutropenic-mouse model in which the *algU* mutant displayed a reduced mean time to death.

TABLE 3. LD_{50}s of strains of *P. aeruginosa* in normal C57BL/6 inbred mice[a]

Strain (genotype)[b]	LD_{50} (CFU)	N[c]
PAO1 (*algU*$^+$ *mucD*$^+$)	1.2×10^6	25
PAO6860 (PAO1 *mucD*::Gm^r)	2.6×10^7	20
PAO6852 (PAO1 *algU*::Tc^r)	1.8×10^5	25

[a] Five mice (5 to 7 weeks old) per dose group, receiving inocula by i.p. injection, were tested as described in Materials and Methods. LD_{50}s were determined by Probit analysis. For confidence intervals, see text.

[b] *mucD* is a part of the *algU mucABCD* gene cluster (4) and encodes a homolog of HtrA (27), a serine protease controlled by σ^E in *E. coli* and required for full virulence in *S. typhimurium* (22).

[c] *N*, number of mice in each group. Mean times to death ranged from 21.4 ± 6.3 to 23.2 ± 8.2 h.

TABLE 4. Mortalities in C3H/HeJ mice challenged with PAO1 and PAO6852

P. aeruginosa strain (genotype)	Infectious dose (CFU)[a]	Mortality[b]
PAO1 (*algU*$^+$)	2.5×10^6	1/1
	1.8×10^6	0/4
	3.5×10^5	0/4
PAO6852 (*algU*::Tc^r)	2.5×10^6	1/1
	1.3×10^6	4/4
	6.5×10^5	2/4

[a] Actual infectious doses administered were determined by plating at the time of injection.

[b] Mortality is expressed as number of mice dead/total number of mice tested. Mice were challenged by i.p. injection, and deaths occurred within 48 h postinfection; no additional fatalities were observed past that time. Calculated LD_{50}s (Probit analysis) were 2.1×10^6 CFU for PAO1 and 6.5×10^5 CFU for PAO6852.

Serum sensitivity, LPS profiles, and virulence in C3H/HeJ mice of *algU*::Tc^r *P. aeruginosa*. The paradoxical effect of *algU* inactivation, which surprisingly increased *P. aeruginosa* systemic virulence, suggested that some changes must have occurred to outweigh the increased sensitivity of *algU* mutants to reactive oxygen species and phagocytic cells observed in vitro. In order to initiate investigations of the potential mechanisms underlying this phenomenon, we compared the growth characteristics, serum sensitivities, lipopolysaccharide (LPS) profiles, and levels of virulence in endotoxin-resistant mice for PAO1 and PAO6852. No differences in growth could be observed on standard media and in heat-inactivated serum (data not shown). Only minor differences in serum sensitivity were detected, with survival rates of 42.9% ± 1.8% for PAO1 and 92.4% ± 11.5% for PAO6852 after 60 min of incubation in 10% pooled human sera (*P* [*t* test], 0.014). The LPS profiles of *algU*$^+$ and *algU*::Tc^r cells, examined by SDS-PAGE, showed no detectable quantitative or qualitative differences (data not shown). Furthermore, when the levels of virulence for *algU*::Tc^r cells and those of the parental *algU*$^+$ strain for the endotoxin-resistant mouse strain C3H/HeJ (53) were compared, the mortalities (Table 4) appeared similar to those observed with C57BL/6 mice.

Expression of a subset of polypeptides is increased in *algU*::Tc^r cells. Since *algU* encodes an alternative sigma factor, its inactivation is likely to have pleiotropic effects in *P. aeruginosa*. In order to test how the inactivation of *algU* affects gene expression in *P. aeruginosa*, we compared profiles of de novo synthesized polypeptides in *algU*$^+$ and *algU*::Tc^r strains under extreme-heat shock conditions (Fig. 3). The patterns of metabolically labeled polypeptides on 2D gels (Fig. 3) indicated that in addition to the previously identified spot (46) corresponding to AlgU (Fig. 3A [polypeptide with apparent molecular mass of 27.5 kDa]), at least five polypeptides were downregulated or absent in PAO6852 (Fig. 3B) compared with those in PAO1 (Fig. 3A). These polypeptides were termed Upp13, Upp22, Upp37, Upp40, and Upp46 (AlgU positively regulated proteins with the indicated molecular masses). Surprisingly, another set of seven polypeptides in PAO6852 were found to be upregu-

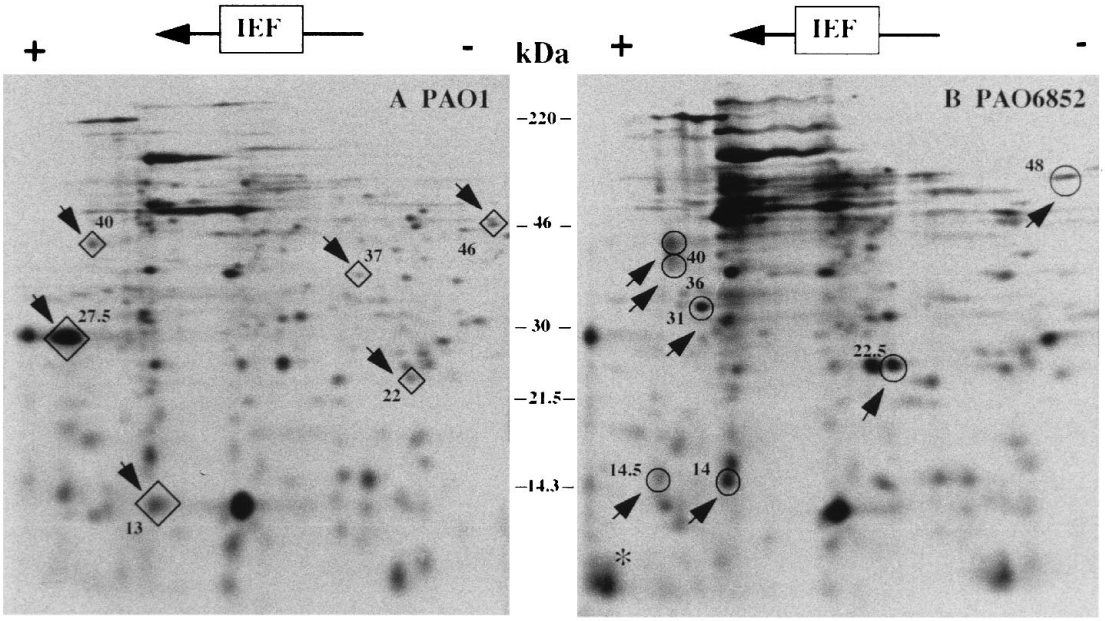

FIG. 3. Inactivation of *algU* has both negative and positive effects on gene expression in *P. aeruginosa*. Metabolically labeled de novo-synthesized polypeptides from PAO1(*algU*$^+$) (A) and PAO6852(*algU*::Tc^r) (B), subjected to extreme heat shock, were analyzed by 2D gel electrophoresis (first dimension, sodium dodecyl sulfate-polyacrylamide gel; second dimension, isoelectric focusing [IEF] gel) and autoradiography. Diamonds and arrows pointing downward, polypeptides (apparent molecular mass of 13, 22, 27.5, 37, 40, and 46 kDa) induced by extreme heat shock in PAO1 that are absent or downregulated under the same conditions in PAO6852; circles and arrows pointing upward, polypeptides (apparent molecular mass of 14, 14.5, 22.5, 31, 36, 40, and 48 kDa) that are upregulated in PAO6852 relative to PAO1; asterisk, a radioactive spot for which no M_r was assigned. The 27.5-kDa polypeptide absent in PAO6852 is AlgU, as shown previously (46). Note that *algU* inactivation has both positive and negative effects on gene expression in *P. aeruginosa.*

lated compared with those in PAO1 (Fig. 3B). The polypeptides derepressed in the absence of AlgU were termed Unp14, Unp14.5, Unp22.5, Unp31, Unp36, Unp40, and Unp48 (AlgU negatively regulated proteins with the indicated molecular masses). The finding that several gene products in *algU*::Tc^r *P. aeruginosa* can be induced suggests a complex role for *algU* as both a positive and a negative regulator of gene expression in *P. aeruginosa*, a phenomenon that could be at least partially related to the observed virulence properties of *algU* mutant cells.

DISCUSSION

In this study, the effects of *algU* inactivation on virulence and sensitivity of *P. aeruginosa* to acellular and cellular microbicidal systems have been analyzed. This study also represents an initial analysis of the role in systemic bacterial virulence of a gene encoding an extreme-stress sigma factor from the AlgU-σ^E family (7, 32, 55). Since close functional or sequence homologs of AlgU (σ^E) are present in other gram-negative pathogens, e.g., *S. typhimurium* (35, 56) and *Haemophilus influenzae* (12), future studies could provide information regarding their potential role in the virulence of these organisms. It is likely that depending upon the differences in lifestyles of pathogens or the types of infections that they cause, σ^E analogs may play different roles. Even in a single organism, e.g., *P. aeruginosa*, the spectrum of *algU* influence on the pathogenic and virulence properties of this bacterium ranges from a positive role in enhancing persistence, as in the case of conversion to mucoidy during chronic infections in CF (8, 38), to apparently negative effects on virulence, as in acute systemic disease as observed in this study. The latter finding parallels, at least in its first approximation, the phenomenon that *P. aeruginosa* bacteremias are seldom encountered in CF; while mucoid *P. aeruginosa* strains with upregulated AlgU are selected in CF lung tissue and can grow to staggering densities, they nevertheless remain confined to the lumen of the respiratory tract (1, 38, 51).

The alternative sigma factor AlgU has been uncovered (32) on the basis of its role as the central regulator of conversion to mucoidy in *P. aeruginosa* (8, 55). In the initial studies indicating a broader role of *algU* in *P. aeruginosa* physiology, we have shown that mutations in *algU* not only cause loss of mucoid phenotype in alginate overproducing strains but also render wild-type nonmucoid *P. aeruginosa* sensitive to high temperatures and redox cycling compounds (35, 56). In these reports, it has also been noted that *algU* has no apparent contribution to defense against exogenously added H_2O_2. We now show that *algU* plays a role in protection against oxidants relevant for the infectious process, e.g., hypochlorite and the microbicidal action of a neutrophil-derived antimicrobial system consisting of myeloperoxidase, an H_2O_2-generating system, and an oxidizable halide cofactor (23, 41). The role of AlgU in resistance to superoxide and hypohalous ions is of potential relevance for the situation in the inflamed CF lung. The chronic infection in CF is notorious for the profuse infiltration of neutrophils which, among other noxious products, release enzymatically generated hypochlorous acid not only into phagosomes but also to the external environment (2, 26, 44). It is thus reasonable to assume that upregulation of *algU*-dependent systems may be beneficial under such conditions and could provide some selective advantage to *P. aeruginosa.*

While the full spectrum of *algU*-dependent functions is not known at present, it is likely that in addition to alginate, which is an effective scavenger of reactive oxygen intermediates (25, 49), other factors are involved in enhancing *P. aeruginosa* resistance to oxidants. This notion is based on the increased sensitivity of nonmucoid *P. aeruginosa* to chemically and enzy-

matically generated hypochlorite when *algU* is inactivated. These considerations are further underscored by the results of the experiment in which *algD* inactivation in the wild-type nonmucoid strain PAO1 did not reduce resistance to hypochlorite (Table 2), indicating that *algU*-controlled products besides alginate are required for full protection against oxidants. For example, *mucD*, which may be under partial control of *algU* (4), is one candidate for such defenses against oxidative stress. However, a complicating factor in the case of *mucD* is its possible role in feedback control of AlgU activity (4, 55), which may be responsible for the inverse sensitivity patterns to specific oxidants in *algU* and *mucD* mutants (4, 35, 56). For instance and in contrast to *algU* mutations, inactivation of *mucD* causes increased sensitivity to H_2O_2 and actually reduces sensitivity to paraquat (4), possibly via induction of AlgU (4, 55). We have also addressed the possibility that superoxide dismutase levels in *algU* mutants might be altered. However, no differences in the activity of the major *P. aeruginosa* superoxide dismutase SodB could be observed on activity gels (data not shown). A major role for the minor dismutase activity, SodA, can be excluded on the basis of the negligible effects of *sodA* inactivation on paraquat sensitivity in *P. aeruginosa* (19), which are far below the effects of *algU* inactivation (35, 56). Further supporting this conclusion is the absence of detectable changes in *algU* mutants within the region corresponding to SodA in activity gels, even under growth conditions enhancing *sodA* expression (19) (data not shown). Future analyses of *algU*-dependent gene expression, as exemplified in this work by 2D gel profiles of metabolically labeled proteins, could aid in the future definition of additional factors controlled by AlgU which contribute to the protection of *P. aeruginosa* against oxidative stress and other bactericidal mechanisms of the host. While Fig. 3 shows a differential display of gene expression under extreme heat shock, it is possible that other stress conditions, known to affect *algU* mutants (4, 35) and relevant for the in vivo situation, cause similar effects.

In contrast to the results in acellular assays, which indicate a significant role for *algU* in the defense against reactive oxygen intermediates, only small differences in susceptibility to the bactericidal action of phagocytic cells have been noted in our experiments. Granulocytes, which play a major role in the control of *P. aeruginosa* infections, kill microorganisms by oxygen-dependent and oxygen-independent pathways, of which the latter mechanism appears to be sufficient for the bactericidal activity against *P. aeruginosa* (31, 37, 52). The capacity of phagocytic cells to control *P. aeruginosa* independently of reactive oxygen intermediates can at least partially explain the relatively small differences in the survival of PAO1 (*algU*$^+$) and PAO6852 (*algU*::Tc^r) detected in assays with neutrophils and macrophages. However, the observed statistically significant differences can still be attributed to the effects of *algU* on *P. aeruginosa* resistance to reactive oxygen species. Even in the reports pointing to the ability of neutrophils to control *P. aeruginosa* by oxygen-independent mechanisms, reactive oxygen intermediates appear to provide a statistically significant contribution on the basis of the data presented in a recent study with neutrophils from patients with chronic granulomatous disease (52). Nevertheless, the alternative that *algU*-dependent products or properties provide defense against bactericidal mechanisms of phagocytic cells other than oxidative killing is equally possible.

The experiments with i.p. challenged neutropenic and normal mice indicate that *algU* activity in wild-type *P. aeruginosa* may interfere with the processes associated with mortality in acute systemic disease. While this phenomenon appears paradoxical, since AlgU and its homologs in other bacteria have been implicated in bacterial defense against environmental stress, it should be noted that *P. aeruginosa* is an opportunistic pathogen not adapted to cause infections in hosts with intact normal defenses. Thus, the observed apparent improvement upon the wild type may be viewed as a possible coincidental interference of AlgU or AlgU-dependent systems with *P. aeruginosa* products or surface properties that may participate in promoting fatal septicemia in mice. While we attempted to address the potential nature of such factors, no striking differences between *algU*$^+$ and *algU* mutant cells were observed when LPS profiles or growth rates in vitro (in conventional media and in heat-inactivated serum) were examined. However, our analyses cannot exclude more subtle changes (e.g., variant O side chain switching, in vivo changes in expression of LPS O side chains, etc.) or a possible enhanced expression of other *P. aeruginosa* toxins or virulence factors. The small but measurable increase of PAO6852 in resistance to human serum may be of significance in this context, but a recent study suggesting that PAO1 may not be sensitive to the action of mouse serum (15) argues against this phenomenon as an explanation for the increased virulence. Significantly, inactivation of *algU* not only caused a loss of expression of certain genes as detected by 2D gel analysis but also increased expression under stress conditions of a significant number of other genes, suggesting the possibility that a factor or factors derepressed in *algU*-null mutants may augment *P. aeruginosa* virulence in models of fatal sepsis in mice.

The phenomena described in this work suggest a function for *algU* in *P. aeruginosa* pathogenesis besides its central role in alginate production. The role of AlgU in defense against reactive oxygen intermediates, which is independent of alginate production, as shown by the comparison of a wild-type parent and its *algU* and *algD* mutant derivatives, could play a significant protective role against such host products present in pathologically excessive amounts in the inflamed CF lung (38, 44). In contrast, any contributions of such systems for the course of acute infection may be eclipsed by the ability of human phagocytic cells to control *P. aeruginosa* chiefly via oxygen-independent mechanisms (31, 37, 52) and by an apparent interference of *algU* expression with the factors or properties contributing to host mortality in systemic disease. In keeping with this trend, it is of interest that the localized endobronchial infection in CF, dominated by mucoid strains with highly active AlgU and characterized by the virtual absence of systemic spread, contrasts the high incidence of hematogenous dissemination of *P. aeruginosa* in non-CF pneumonia (39). Previous investigations with CF strains have shown that these clinical isolates display reduced systemic virulence (29) because of gross changes such as the loss of LPS O side chains (18), the accompanied increase in serum sensitivity, reduced motility (30), and other drastic alterations occurring in infecting strains over many years of selective pressures in the CF lung (8). Our observations, made with genetically defined isogenic strains, suggest that upregulation of the systems controlling alginate production may have a direct contribution to reduced acute virulence of CF strains. This may be of potential significance during the process of the establishment of permanent colonization in CF, which according to some authors coincides with the conversion to mucoidy (21, 24, 38).

In contrast to the processes that dominate acute disease, it is likely that *algU*-dependent functions play a significant role in the establishment of chronic infection in the CF lung. This assumption is based not only on the conversion to mucoidy (6) but may possibly be due to the role of *algU* in resistance to oxidative stress in the wild-type nonmucoid strains which are the initial colonizers of the CF lung (10, 17, 21). Experiments

are under way in our laboratory to examine contributions of *algU* in the initial colonization of the lung in a model of aerosol exposure in normal and CF mice. These studies may reveal a role for *algU*-dependent systems in the establishment of infection and its persistence and could also answer additional questions of how the enhanced expression of the stress response systems controlled by AlgU affects inflammatory processes and the pathology of the lung.

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