The Sigma Factor AlgU (AlgT) Controls Exopolysaccharide Production and Tolerance towards Desiccation and Osmotic Stress in the Biocontrol Agent *Pseudomonas fluorescens* CHA0

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A variety of stress situations may affect the activity and survival of plant-beneficial pseudomonads added to soil to control root diseases. This study focused on the roles of the sigma factor AlgU (synonyms, AlgT, RpoE, and σ^{22}) and the anti-sigma factor MucA in stress adaptation of the biocontrol agent *Pseudomonas fluorescens* CHA0. The *algU-mucA-mucB* gene cluster of strain CHA0 was similar to that of the pathogens *Pseudomonas aeruginosa* and *Pseudomonas syringae*. Strain CHA0 is naturally nonmucoid, whereas a *mucA* deletion mutant or *algU*-overexpressing strains were highly mucoid due to exopolysaccharide overproduction. Mucoidy strictly depended on the global regulator GacA. An *algU* deletion mutant was significantly more sensitive to osmotic stress than the wild-type CHA0 strain and the *mucA* mutant were. Expression of an *algU*'-'*lacZ* reporter fusion was induced severalfold in the wild type and in the *mucA* mutant upon exposure to osmotic stress, whereas a lower, noninducible level of expression was observed in the *algU* mutant. Overexpression of *algU* did not enhance tolerance towards osmotic stress. AlgU was found to be essential for tolerance of *P. fluorescens* towards desiccation stress in a sterile vermiculite-sand mixture and in a natural sandy loam soil. The size of the population of the *algU* mutant declined much more rapidly than the size of the wild-type population at soil water contents below 5%. In contrast to its role in pathogenic pseudomonads, AlgU did not contribute to tolerance of *P. fluorescens* towards oxidative and heat stress. In conclusion, AlgU is a crucial determinant in the adaptation of *P. fluorescens* to dry conditions and hyperosmolarity, two major stress factors that limit bacterial survival in the environment.

Some strains of fluorescent pseudomonads colonize the roots of many crop plants and protect them from diseases caused by soilborne fungal pathogens. Disease suppression by these bacteria involves a blend of mechanisms, including effective competition for nutrients and colonization sites, pathogen inhibition by production of antimicrobial compounds, and induction of resistance in the plant (3, 24, 62). Following introduction into soil, the biocontrol performance of pseudomonads depends largely on their ability to maintain stable populations and to be metabolically active at least over the period needed to exert their beneficial effects. However, in soil these bacteria are exposed to a range of variable biotic and abiotic stress factors, such as competition, predation, and changes in temperature, osmolarity, and availability of water and nutrients (42, 63). Therefore, the sizes of introduced pseudomonad populations may decline considerably within a few weeks, and biocontrol activity often tends to be variable (24, 62).

To ensure survival in changing environments, bacteria rely on regulatory mechanisms that allow them to respond rapidly to stress situations (60). Regulatory elements that make essential contributions to bacterial survival under stress conditions include the alternative sigma factors RpoS (σ^s) and RpoE (σ^{22} ;

also referred to as AlgU or AlgT in fluorescent pseudomonads). RpoS is required for tolerance of stationary-phase cultures of different *Pseudomonas* species towards hyperosmolarity, high temperatures, and agents generating reactive oxygen intermediates (ROIs) (23, 52, 61). AlgU contributes to tolerance towards osmotic, oxidative, and heat stresses in the pathogens *Pseudomonas aeruginosa* (32, 54, 56, 70) and *Pseudomonas syringae* (26).

The extracytoplasmic function sigma factor AlgU is encoded by the *algU* gene, which is part of a highly conserved operon in gram-negative bacteria (19, 26, 35, 44). The function of *algU* has been extensively studied in *P. aeruginosa* with respect to its roles in stress response and in regulation of biosynthesis of the exopolysaccharide (EPS) alginate. Regulation of AlgU activity in *P. aeruginosa* is complex. AlgU positively regulates its own transcription (12, 22, 56, 68). Located downstream of *algU*, the *mucABCD* genes ensure tight control of AlgU activity in *P. aeruginosa* (19). The *mucA* gene encodes a transmembrane protein which acts as an anti-sigma factor for AlgU, and *mucB* codes for a periplasmic protein which is another negative regulator of AlgU (36, 57, 69). MucC and MucD modulate *algU* expression, but the precise functions of these proteins have not been fully established yet (5, 6). Binding of AlgU to the inner membrane protein MucA occurs when MucA interacts with the periplasmic MucB protein (36, 44, 50). Environmental stress conditions are thought to destabilize the MucB-MucA-AlgU complex, leading to release of AlgU into the cytosol. As a consequence, AlgU becomes active and transcription of al-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or phenotype ^a	Reference or source
<i>P. fluorescens</i> strains		
CHA0	Wild type	65
CHA0-Rif	Spontaneous Rif ^r derivative of CHA0	45
CHA89	<i>gacA</i> ::Ω-Km, Km ^r	29
CHA211	<i>mucA</i> ::Tn5, Km ^r	This study
CHA212	Δ <i>algU</i>	This study
CHA212-Rif	CHA0-Rif Δ <i>algU</i> , Rif ^r	This study
CHA213M	Δ <i>mucA</i>	This study
CHA213M. <i>gacA</i>	Δ <i>mucA gacA</i> ::Ω-Km, Km ^r	This study
CHA510	<i>gacS</i> ::Tn5, Km ^r	8
<i>P. aeruginosa</i> strains		
PAO1	Wild type	ATCC 15692
PAO568	<i>mucA2 leu-38 str-2</i> FP2 ⁺	32
PAO6852	PAO1 <i>algU</i> ::Tc ^r , Tc ^r	32
<i>E. coli</i> strains		
DH5α	<i>recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1 Δ(lacZYA-argF)U169 (φ80dlacZΔM15)</i>	51
W3110	Prototroph, sup ⁰ λ ⁻	51
Plasmids		
pBluescript II KS+	Cloning vector, ColE1 replicon, Ap ^r	Stratagene
pLG221	Suicide vector, Coll1 <i>drd-1</i> ::Tn5, IncIα, Km ^r	7
pME497	Mobilizing plasmid, IncP-1, Tra RepA(Ts), Ap ^r	66
pME3049	Suicide vector, ColE1 replicon, RK2-Mob, Hg ^r Km ^r	53
pME3087	Suicide vector, ColE1 replicon, RK2-Mob, Tc ^r	65
pME3088	Suicide vector, ColE1 replicon, RK2-Mob, Tc ^r	65
pME3089Km	pME3088 carrying <i>gacA</i> of <i>P. fluorescens</i> disrupted by an Ω-Km cassette, Tc ^r Km ^r	29
pME6000	Cloning vector, pBBR1MCS derivative, 18 copies per chromosome equivalent in <i>P. fluorescens</i> , Tc ^r	38
pME6010	Cloning vector, pACYC177-pVS1 shuttle vector, 6 copies per chromosome equivalent in <i>P. fluorescens</i> , Tc ^r	21
pME6010ΔB	pME6010 with deletion of the unique <i>Bam</i> HI site, Tc ^r	This study
pME6200B	pME3049 with a 0.8-kb genomic DNA fragment of <i>P. fluorescens</i> CHA211	This study ^b
pME6200X	pME3049 with a 4.5-kb genomic DNA fragment of <i>P. fluorescens</i> CHA211	This study ^b
pME6202X	pME3049 with a 14.5-kb genomic DNA fragment of <i>P. fluorescens</i> CHA0	This study ^b
pME6202XΔEV	pME6202X with a 9.8-kb <i>Eco</i> RV deletion	This study ^b
pME6217	pME6000 with a 2.6-kb <i>Apa</i> I- <i>Bam</i> HI fragment of pME6202XΔEV, containing <i>algUmucA</i>	This study ^b
pME6219	pME6010ΔB with a 2.6-kb <i>Apa</i> I- <i>Bam</i> HI fragment of pME6202XΔEV in which the 275-bp <i>Not</i> I- <i>Cl</i> aI fragment in <i>algU</i> is replaced by the 56-bp <i>Not</i> I- <i>Cl</i> aI polylinker of pBluescript II KS+, contains intact <i>mucA</i>	This study ^b
pME6220	pME6000 with a 1.7-kb <i>Apa</i> I- <i>Msc</i> I fragment of pME6202XΔEV, containing <i>algU</i>	This study ^b
pME6221	pME6010ΔB with a 1.7-kb <i>Apa</i> I- <i>Msc</i> I fragment of pME6202XΔEV, containing <i>algU</i>	This study ^b
pME6222	pME6010ΔB with a 1.0-kb <i>Apa</i> I- <i>Not</i> I fragment of pME6202XΔEV and the 3-kb <i>Bam</i> HI- <i>Dra</i> I fragment of pNM482 containing ' <i>lacZ</i> , <i>algU</i> '-' <i>lacZ</i> translational fusion at the <i>Not</i> I site in <i>algU</i>	This study ^b
pME6551	Cloning vector, pME6000 derivative with insertion of the Gm ^r determinant (<i>aacI</i> gene) of pML8 at the <i>Eco</i> RV site in the <i>tetA</i> gene, Tc ^s Gm ^r	This study
pME6555	pME6551 with a 1,712-bp <i>Sau</i> 3A fragment of pME6215 (5' end located 48 bp upstream of the <i>algU</i> start codon) containing <i>algUmucA</i> placed under the control of the <i>lac</i> promoter	This study ^b
pML8	RSF1010-derived cloning vector carrying the Gm ^r gene <i>aacI</i> , Tc ^r Gm ^r	28
pNM482	ColE1 replicon, ' <i>lacZ</i> , Ap ^r	43
pUK21	Cloning vector, <i>lacZ</i> α, Km ^r	64

^a Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Hg^r, mercury resistance; Km^r, kanamycin resistance; Rif^r, rifampin resistance; Tc^r, tetracycline resistance.

^b See Fig. 1.

ginate biosynthesis and other genes occurs. Any change in the balance of this regulatory system influences the titer of available active AlgU. For example, mutations in *mucA* or *mucB* cause increased activity of AlgU, which leads to mucoidy due to overproduction of alginate (33, 34, 36). Mucoid conversion due to spontaneous lesions in *mucA* is typically detected in *P. aeruginosa* isolates from chronically infected cystic fibrosis patients, and the production of copious amounts of alginate is thought to be important for virulence and survival of these bacteria in the lungs (4, 34).

Little is known about the role of AlgU and its negative regulators in plant-beneficial pseudomonads. In the present study, we identified a genomic region which comprises the *algU-mucA-mucB* gene cluster in *Pseudomonas fluorescens* CHA0, a well-characterized soil bacterium with broad-spectrum biocontrol activity (24, 65). We found that AlgU, along

with *MucA*, tightly controls EPS biosynthesis and tolerance towards osmotic stress in this bacterium. In contrast to AlgU of pathogenic pseudomonads, AlgU of strain CHA0 does not contribute to survival in response to treatment with heat and ROIs. Finally, we present the first evidence that AlgU is a crucial determinant in adaptation of *P. fluorescens* to desiccation stress, a major factor that limits bacterial survival in formulations or in soil.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. *P. fluorescens* strains were cultivated on nutrient agar (NA) (59), on King's medium B (KMB) agar (27), in nutrient yeast broth (NYB) (59), in KMB broth, and in Luria-Bertani broth (LB) (51) at 30°C with aeration. *Escherichia coli* and *P. aeruginosa* strains were grown on NA and in NYB at 37°C. Antimicrobial compounds, when required, were added to the growth media at the following concentrations: ampicillin, 100

FIG. 1. Physical location of the *nadB*, *algU*, *mucA*, and *mucB* genes in *P. fluorescens* CHA0. Symbols: ▼, Tn5 insertion in the chromosome of strain CHA211; Δ, region deleted in strains CHA212 and CHA213M and in plasmid pME6219. The shaded arrows indicate the genes sequenced or partly sequenced on the *EcoRI*-*Bam*HI fragment of pME6202XΔEV. The open boxes indicate the genomic inserts in ColE1-based plasmids pME6200B, pME6200X, pME6202X, and pME6202XΔEV. The lines indicate the fragments cloned into vector pME6000 to obtain pME6217, pME6220, and pME6555 and into vector pME6010ΔB to obtain pME6219, pME6221, and pME6222.

μg/ml; chloramphenicol, 25 μg/ml; HgCl₂, 20 μg/ml; gentamicin, 10 μg/ml; kanamycin sulfate, 25 μg/ml; rifampin, 100 μg/ml; and tetracycline hydrochloride, 25 μg/ml for *E. coli* and 125 μg/ml for *P. fluorescens* strains. When appropriate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was incorporated into solid media to monitor β-galactosidase expression (51).

DNA manipulation and sequencing. Small-scale plasmid DNA preparations were obtained from *P. fluorescens* strains and pME3049-, pME3087-, and pME3088-based plasmids were isolated from *E. coli* by the alkaline lysis method (51). All other plasmids were prepared from *E. coli* by the method of Del Sal et al. (10). Qiagen-tip 100 columns (Qiagen Inc.) were used for large-scale plasmid DNA preparation. Chromosomal DNA of *P. fluorescens* was isolated as described by Schneider et al. (53). Standard techniques were used for restriction, agarose gel electrophoresis, dephosphorylation, generation of blunt ends with the Klenow fragment of *E. coli* DNA polymerase I or T4 DNA polymerase (Roche), isolation of DNA fragments from low-melting-point agarose gels, and ligation (51). Restriction fragments were purified from agarose gels with a GeneClean II kit (Bio 101). Bacterial cells were transformed with plasmid DNA by CaCl₂ treatment (51) or electroporation (15). Southern blotting with Hybond N membranes (Amersham), random-primed DNA labeling with digoxigenin-11-dUTP, hybridization, and detection (Roche) were performed by using the protocols of the suppliers. Subclones of pME6202XΔEV (Table 1; Fig. 1) constructed in pBluescript II KS+ were used for nucleotide sequence determination.

Both strands of the 2,825-bp *EcoRI*-*Bam*HI fragment of pME6202XΔEV were sequenced by the dideoxy chain termination method using a Sequenase 2.0 kit (United States Biochemical, Cleveland, Ohio) and T7 polymerase from Pharmacia. Nucleotide and deduced amino acid sequences were analyzed with programs of the University of Wisconsin Genetics Computer Group package (version 9.1).

Plasmid mobilization and transposition. Derivatives of the suicide plasmids pME3049, pME3087, and pME3088 were mobilized from *E. coli* to *P. fluorescens* with helper plasmid pME497 in triparental matings as described by Schneider et al. (53). Transposon mutagenesis in which *E. coli* W3110 containing Tn5 suicide plasmid pLG221 (7) was used as the donor strain and *P. fluorescens* CHA0 was used as the recipient strain was carried out as described previously (37).

Construction of *P. fluorescens* mutants by gene replacement. For construction of the *algU* in-frame mutant CHA212 (Fig. 1), the 275-bp *NotI*-*ClaI* fragment in *algU* was replaced by the 56-bp *NotI*-*ClaI* polylinker from pBluescript II KS+. The flanking genomic DNA, consisting of the 1.2-kb *HincII*-*NotI* fragment and the 1.4-kb *ClaI*-*Bam*HI fragment of pME6202XΔEV (Fig. 1), was cloned into suicide vector pME3087 (65). To obtain the *mucA* in-frame mutant CHA213M (Fig. 1), the 309-bp *SmaI*-*MscI* fragment in *mucA* was deleted. The flanking genomic DNA, consisting of the 0.6-kb *PvuII*-*SmaI* fragment and the 0.8-kb *MscI*-*Bam*HI fragment of pME6202XΔEV, was cloned into suicide vector pME3088 (65). The derivatives of the suicide plasmids, which carried a tetracy-

cline resistance determinant, were mobilized with helper plasmid pME497 (66) to wild-type strain CHA0. Cells with a chromosomally integrated plasmid were selected for tetracycline resistance. Excision of the vector by a second homologous recombination event was observed after enrichment for tetracycline-sensitive cells (53). The same approach was used to create an *algU* in-frame mutation in rifampin-resistant strain CHA0-Rif. To obtain the *muca* *gacA* double mutant CHA213M, plasmid pME3089Km, a derivative of suicide vector pME3088 carrying *gacA* disrupted by an Ω -Km cassette (29), was mobilized into strain CHA213M. Selection of cells with a chromosomally integrated plasmid and excision of the vector by a second crossover were performed as described above. Selection for kanamycin resistance ensured the presence of the Ω -Km insertion in strain CHA213M. The *algU*, *muca*, and *gacA* mutations were checked by Southern blotting (data not shown).

Extraction and quantification of EPS from *P. fluorescens*. Aliquots (100 μ l) of an overnight LB culture of strain CHA0 or one of its derivatives were plated on KMB agar plates. Three replicate plates per strain were prepared and incubated for 24 h at 30°C. Extraction and quantification of EPS were performed by using a procedure described by May and Chakrabarty (39). Briefly, cells were removed from the medium and suspended in 0.9% NaCl. The suspensions were centrifuged for 30 min at $17,700 \times g$ and 4°C to separate the cells from the EPS. The cell pellets were kept for determinations of total cell protein contents. The EPS was repeatedly precipitated and washed with ice-cold absolute ethanol. The contents of uronic acid polymers (components of alginate) in the samples were then assessed by performing the colorimetric carbazole assay (39), using D-mannuronic acid (Sigma Chemical Co., St. Louis, Mo.) and alginic acid from seaweed (*Macrocystis pyrifera*; Sigma) as the standards. The uronic acid content was expressed per milligram of total cell protein as determined by the Lowry method, using bovine serum albumin as the standard.

Purification and biochemical analysis of EPS. Mucoid layers from five KMB agar cultures of highly mucoid mutant strains CHA211 and CHA213M, cultivated under the conditions described above, were scraped off the plates with a sterilized spatula, pooled, and suspended in 50 ml of phosphate-buffered saline (pH 7.2). After vigorous stirring at 4°C for 1 h, the viscous solution was centrifuged at $17,700 \times g$ and 4°C for 4 h to remove the bacterial cells. The remaining proteins in the supernatant were denatured by heating at 80°C for 30 min and removed by centrifugation at $17,700 \times g$ for 30 min. Precipitation of EPS by the addition of ice-cold absolute ethanol, washing, and removal of nucleic acids by digestion with DNase I (type IV; Sigma) and RNase A (type 1A; Sigma) were performed as described by Pedersen et al. (47). The EPS was then further purified by ion-exchange chromatography. Samples were dissolved in 25 mM ammonium carbonate, loaded onto a DEAE-Sepharose CL-6B column (1.6 by 20 cm; bed volume, 40 ml; Pharmacia), which had been equilibrated with the same buffer, and eluted with a linear 25 mM to 1 M ammonium carbonate gradient (39, 47). Fractions (5 ml) containing uronic acids were pooled, extensively dialyzed against sterile water, and lyophilized.

The total carbohydrate of the purified EPS was quantified colorimetrically by performing the phenol-sulfuric acid assay (13) with 3% (wt/vol) phenol, using D-mannuronic acid lactone (Sigma) and seaweed alginate as the standards. The degree of acetylation of uronic acids was assessed on the basis of a colorimetric reaction with hydroxylamine hydrochloride (40), using β -D-glucose-pentaacetate (Sigma) as the standard.

Effect of osmotic stress on *algU*'-'*lacZ* expression and growth. *P. fluorescens* CHA0 and its mutant derivatives carrying an *algU*'-'*lacZ* translational fusion on plasmid pME6222 (Table 1; Fig. 1) were grown in 20 ml of KMB without selective antibiotics in 100-ml Erlenmeyer flasks plugged with cotton. Plasmid pME6222 carries the *algU* promoter region and the 5' end of *algU* fused to '*lacZ*' from pMN482 (43). To induce osmotic stress, KMB was supplemented with NaCl (0.6, 0.8, 1.0, or 1.2 M) or sorbitol (1.2 or 1.6 M), a nonionic solute which cannot be metabolized by strain CHA0. For inoculation, aliquots of exponential-growth-phase LB cultures of the bacterial strains were used to adjust the cell concentrations to an optical density at 600 nm (OD_{600}) of 0.05. Cultures were incubated with rotational shaking (180 rpm) at 30°C. β -Galactosidase specific activities of at least three independent cultures were monitored by the Miller method (51).

Doubling times of strain CHA0 and its derivatives (cultivated as described above) were calculated from OD_{600} values between 0.05 and 1.5 (i.e., during exponential growth). After 24 h of incubation, bacterial survival was assessed by plating serial dilutions of the cultures on NA and determining the ratio of CFU from osmotically stressed cultures to CFU from nonstressed cultures.

Desiccation survival assay performed with filter disks. The sensitivity of *P. fluorescens* to desiccation on filters was assessed by using a modification of the procedure described by Ophir and Gutnick (46). Dilutions of overnight bacterial cultures were vacuum filtered onto Millipore filters (no. HAWP04700; pore size, 0.45 μ m; diameter, 3.5 cm) in order to obtain about 10 to 20 physically separated

bacterial cells per filter. The filters were placed onto KMB agar plates and incubated at 30°C for 24 h, which yielded about 5×10^7 CFU per colony that had developed from the individual bacterial cells. Bacterial colonies were then slowly dried by removing the filters from the agar plates, cutting the filters into small pieces so that each piece contained a single bacterial colony, and incubating the pieces in empty petri dishes at 30°C for 24 h. Colonies on filter pieces that were placed on agar medium lacking nutrients (18 g of Serva agar per liter, 1.15 g of K_2HPO_4 per liter, 1.5 g of $MgSO_4 \cdot 7H_2O$ per liter) and were incubated for the same period served as controls. Cells from a single colony on each filter were then suspended in 1 ml of a 0.85% NaCl solution by vigorous mixing with a Vortex mixer for 15 min, and serial dilutions were plated on NA to determine the number of CFU per colony. Washed filter pieces incubated on NA plates showed that almost all cells were removed by this treatment. The level of survival was calculated by determining the percentage of the number of CFU in desiccated colonies relative to the number of CFU in control colonies. Each filter piece was handled separately, and the numbers of CFU were determined for at least five colonies per treatment. The experiment was repeated three times.

Desiccation survival assay performed with soil microcosms. Survival of bacterial strains was monitored in desiccating, sterile, artificial soil and in desiccating, nonsterile, natural soil. The artificial soil consisted of pure vermiculite (expanded with 30% H_2O_2), quartz sand with different sizes of particles, and quartz powder mixed at a ratio of 10:70:20 (by weight) and moistened with 10% (wt/wt) distilled water (25). The artificial soil was autoclaved twice prior to bacterial inoculation. Natural sandy loam soil was collected from the surface horizon of a Swiss cambisol located at Eschikon near Zurich (45). The soil was sieved through a 5-mm mesh screen prior to use, and stones and roots were removed. The bacterial cell suspensions used in microcosms were prepared from exponential-growth-phase cultures grown in LB at 24°C for 16 h. The cells were washed twice in sterile distilled water, and the cell concentration was adjusted to an OD_{600} of 0.2. For inoculation of the artificial or natural soil, 67 ml of the bacterial suspension was thoroughly mixed into 1,000 g of soil with a sterilized spoon to obtain about 10^7 CFU per g of soil. For natural soil microcosms, rifampin-resistant derivatives of the bacterial strains were used as the inoculants. Aliquots (20 g) of inoculated soil were placed into sterile 200-ml Erlenmeyer flasks with wide openings. The openings were covered with one layer of sterilized paper cloth, which allowed slow evaporation of the soil water. Control microcosms kept at a constant soil moisture level were prepared by placing 200-g portions of sterile, artificial soil or nonsterile, natural soil containing the bacterial inoculum into 500-ml bottles, which then were sealed hermetically. The initial water content of the artificial soil after addition of bacteria was $15.2\% \pm 0.2\%$, which corresponded to a soil water potential (Ψ_w) of about -0.01 MPa. Natural soil contained $26.9\% \pm 0.2\%$ water, which corresponded to a Ψ_w of about -0.02 MPa. The soil microcosms were incubated in the dark at 24°C with 65% relative humidity. For sampling and enumeration of the bacterial inoculants, the entire contents of a flask with desiccated soil or a 5-g sample from a control soil kept at a constant humidity was suspended in sterile distilled water by vigorous agitation on a shaker at 260 rpm for 20 min. At each time point, the number of cultivable cells and the soil water content were determined for three replicate flasks or soil samples per treatment. The numbers of CFU were determined by plating serial dilutions of the soil suspensions on NA. Rifampin-resistant derivatives were recovered from natural soil by plating samples on NA containing 100 μ g of rifampin per ml. No rifampin-resistant background bacterial population was present in the natural soil. CFU data were expressed per gram (dry weight) of soil and were $\log_{10}$ transformed before means and standard deviations were calculated. Water content was assessed by oven drying soil samples at 105°C to constant weight. The Ψ_w of soil was determined by using a filter paper method described by McInnes et al. (41).

Susceptibility to oxidative stress. Sensitivity to paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride; Sigma), hydrogen peroxide (H_2O_2), or sodium hypochlorite (NaOCl) was examined as described by Martin et al. (32). Filter disks (diameter, 6 mm; Millipore) were soaked with 10 μ l of paraquat (1.9 or 3.8%, wt/vol), H_2O_2 (3 or 12%, vol/vol), or NaOCl (5 or 10%, vol/vol) and placed on a layer of soft agar (2 ml of NYB with 0.8% agar) containing 100 μ l of a *P. fluorescens* or *P. aeruginosa* overnight culture covering NA. In another approach, disks (diameter, 10 mm) were soaked with 50- μ l portions of the agents mentioned above. The diameters of the inhibition zones surrounding the impregnated disks were measured after overnight incubation at 30°C for *P. fluorescens* strains and at 37°C for *P. aeruginosa* strains.

Sensitivity to high temperatures. *P. fluorescens* CHA0 and mutant derivatives of this strain were grown at 30°C in 20 ml of KMB or NYB in 100-ml Erlenmeyer flasks sealed with cellulose stoppers. When the cultures reached an OD_{600} of 0.5, the flasks were transferred to a water bath and incubated for 0, 5, 10, 20, 30, 60, and 90 min at 42 or 48°C with rotational shaking at 180 rpm. At each time point,

three replicate cultures were sampled to determine the number of CFU on NA. CFU were counted after incubation for 24 and 72 h at room temperature. Levels of survival were expressed as percentages of the input number of CFU at time zero.

Nucleotide sequence accession number. The nucleotide sequence of the *nadB'*, *algU*, *mucA*, and *mucB'* genes of *P. fluorescens* CHA0 has been deposited in the GenBank database under accession no. AF399758.

RESULTS

Isolation and characterization of mucoid mutant CHA211. Following Tn5 mutagenesis of *P. fluorescens* strain CHA0, 1 of the 1,000 mutants tested had a highly mucoid colony phenotype when it was grown on KMB or NA. In contrast, wild-type strain CHA0 is naturally nonmucoid. The mucoid phenotype of the mutant strain, CHA211, was stable, even when the strain was repeatedly subcultured in nonselective media for more than 1 week. Southern hybridization confirmed that mutant CHA211 contained a single Tn5 insertion (data not shown). The Tn5 insertion was located 550 bp downstream of the initiation codon of the *mucA* gene (Fig. 1 and see below).

Cloning and sequence analysis of the genes surrounding the Tn5 insertion in strain CHA211. To localize the genomic region mediating mucoidy in strain CHA211, a two-step Tn5-directed cloning strategy (53) was used to clone the wild-type genes corresponding to the genes that were inactivated by the Tn5 insertion in the mutant. Suicide vector pME3049 carrying the kanamycin resistance determinant of Tn5 was mobilized into strain CHA211. Integration of pME3049 into the chromosome occurred at a frequency of 7.2×10^{-7} per donor. Chromosomal DNA of CHA211::pME3049 was digested with *Bam*HI or *Xba*I, ligated, and used to transform *E. coli* DH5 α . The resulting plasmids, pME6200B and pME6200X, carried 0.8- and 4.5-kb genomic DNA inserts, respectively, downstream of the Tn5 insertion site (Fig. 1). For isolation of the wild-type genes, plasmid pME6200B was transferred into wild-type strain CHA0. Digestion of genomic DNA of CHA0::pME6200B with *Xho*I, self-ligation, and transformation of *E. coli* resulted in plasmid pME6202X (Fig. 1) carrying a 14.6-kb genomic fragment. To reduce the size of the insert in pME6202X, the plasmid was digested with *Eco*RV and ligated. The plasmid obtained, pME6202X Δ EV (Fig. 1), contained a 4.7-kb genomic DNA fragment.

The 2,825-bp nucleotide sequence of the *Eco*RI-*Bam*HI genomic fragment in pME6202X Δ EV (Fig. 1, expanded region) revealed that there were two complete and two partial open reading frames, designated *nadB'*, *algU*, *mucA*, and *mucB'*, by analogy with homologous genes in *P. aeruginosa* (12, 31, 33), *P. syringae* pv. *syringae* (26), and *Azotobacter vinelandii* (35). The proposed start codons of *nadB'*, *algU*, *mucA*, and *mucB'* of *P. fluorescens* CHA0 are preceded by plausible ribosome-binding sites. The deduced product (193 amino acids, 22.2 kDa) of the *algU* gene of *P. fluorescens* CHA0 is very similar to alternative sigma factor AlgU (σ^{22}) of *P. syringae* pv. *syringae* (accession no. AF190580; 97% identity), *A. vinelandii* (accession no. U22895; 93% identity), and *P. aeruginosa* (accession no. L04794 and L36379; 91% identity) and is also related to RpoE of *E. coli* (accession no. EC37089; 63% identity). The *mucA* gene is located downstream of *algU*, and its product (195 amino acids, 20.9 kDa) is 82, 74, and 63% identical to the MucA anti- σ^{22} factor of *P. syringae* pv. *syringae*

TABLE 2. EPS production by *P. fluorescens* CHA0 and derivatives of this strain

Strain ^a	Genotype	Phenotype	EPS production (μ g/mg of protein) ^b
CHA0	Wild type	Nonmucoid	42 d
CHA0/pME6000	Wild type/— ^c	Nonmucoid	54 d
CHA0/pME6220	Wild type/ <i>P</i> _{alg} - <i>algU</i> ⁺	Mucoid	4,602 c
CHA0/pME6217	Wild type/ <i>P</i> _{alg} - <i>algU</i> ⁺ <i>mucA</i> ⁺	Mucoid	6,365 c
CHA0/pME6555	Wild type/ <i>P</i> _{lac} - <i>algU</i> ⁺ <i>mucA</i> ⁺	Mucoid	14,802 b
CHA212	Δ <i>algU</i>	Nonmucoid	77 d
CHA212/pME6010 Δ B	Δ <i>algU</i> /—	Nonmucoid	70 d
CHA212/pME6221	Δ <i>algU</i> / <i>algU</i> ⁺	Nonmucoid	21 d
CHA211	<i>mucA</i> ::Tn5	Mucoid	28,143 a
CHA213M	Δ <i>mucA</i>	Mucoid	4,123 c
CHA213M/pME6010 Δ B	Δ <i>mucA</i> /—	Mucoid	4,464 c
CHA213M/pME6219	Δ <i>mucA</i> / <i>mucA</i> ⁺	Nonmucoid	61 d
CHA213M.89	Δ <i>mucA</i> <i>gacA</i> :: Ω -Km	Nonmucoid	54 d
CHA89	<i>gacA</i> :: Ω -Km	Nonmucoid	43 d

^a Bacteria were grown on KMB agar plates at 30°C for 24 h. Plasmid pME6221 was derived from the low-copy-number vector pME6010 (six copies per chromosome equivalent) (21); plasmids pME6217, pME6220, and pME6555 were derivatives of the multiple-copy-number vector pME6000 (18 copies per chromosome equivalent) (38). *P*_{alg} and *P*_{lac} indicate control from the natural promoter and the *lac* promoter, respectively.

^b EPS production was measured in micrograms of uronic acid produced per milligram of total cell protein. The data are means based on three individually assessed cultures. Values followed by different letters are significantly different at $P = 0.05$ (Student's *t* test).

—, empty vector.

(accession no. AF190580), *P. aeruginosa* (accession no. L14760 and L36379), and *A. vinelandii* (accession no. U22660), respectively. The product of the adjacent, incompletely sequenced *mucB* gene is 66, 61, and 65% identical to MucB (AlgN) of *P. syringae* pv. *syringae* (accession no. AF190580), *P. aeruginosa* (accession no. L14760), and *A. vinelandii* (accession no. U22660), respectively. The intergenic region upstream of *algU* of *P. fluorescens* comprises 558 nucleotides and exhibits only 48% nucleotide identity with the corresponding region of *P. aeruginosa*. Two putative AlgU (RpoE) recognition sites were found 60 bp (GAAGTT-16 nucleotides-TCTAT) and 253 bp (GAAGTT-17 nucleotides-TCAAT) upstream of the translational start site of *algU*. Remarkably, the location and sequence of the first AlgU recognition site (60 bp upstream of the initiation codon of *algU*) are conserved in *P. fluorescens*, *P. syringae* pv. *syringae* (26), and *P. aeruginosa* (56). The location and sequence of the second AlgU recognition site are almost identical in *P. fluorescens* and *P. syringae* pv. *syringae*. The divergently oriented *nadB'* gene upstream of *algU* was sequenced only in its 5' region. The deduced product (117 amino acids) exhibits similarities to N termini of the L-aspartate oxidase for NAD biosynthesis of *P. aeruginosa* (accession no. U17232; 82% identity) and *E. coli* (accession no. X12714; 56% identity). Based on these sequence comparisons, it appears that the arrangement of the *nadB*, *algU*, *mucA*, and *mucB* genes is conserved in *P. fluorescens*, *P. syringae*, *P. aeruginosa*, and *A. vinelandii*.

EPS production in *P. fluorescens* is controlled by the AlgU regulon. Chromosomal *algU* and *mucA* in-frame deletion mutations were created in strain CHA0 (Fig. 1) as described in Materials and Methods, and the mutants were tested for EPS production by using the carbazole assay for uronic acids. The nonmucoid strains CHA0 (wild type) and CHA212 (*algU* mu-

FIG. 2. Kinetics of expression of an *algU'*-*lacZ* fusion in response to NaCl stress. β-Galactosidase activity was assayed in *P. fluorescens* CHA0 (●) and *ΔalgU* mutant CHA212 (○), both carrying pME6222. Growth of CHA0 (▲) and growth of CHA212 (△) were determined by measuring OD₆₀₀. Strains were grown at 30°C in KMB without (A) or with (B) 0.6 M NaCl. The data are means ± standard deviations based on three experiments. Some of the error bars were too small to be included.

tant) produced very low levels of EPS after incubation on KMB agar for 24 h (Table 2). In contrast, *mucA* in-frame deletion mutant CHA213M developed a mucoid phenotype on KMB agar and produced copious amounts of EPS (Table 2). The *mucA*::Tn5 mutant CHA211 (Fig. 1) produced even more mucoid material than the *mucA* in-frame deletion mutant CHA213M produced (Table 2). This may have been due to a polar effect of the Tn5 insertion on other *AlgU* regulatory genes downstream of *mucA*, especially *mucB* (36, 50). The level of EPS production in mucoid mutant CHA213M could be restored to the low wild-type level by complementation with *mucA*⁺ plasmid pME6219 (Fig. 1), whereas introduction of the cloning vector pME6010ΔB alone had no effect (Table 2). Complementation of the *ΔalgU* mutation in CHA212 by pME6221 carrying intact *algU*⁺ (Fig. 1) did not significantly affect the nonmucoid phenotype of the strain (Table 2).

When *AlgU* was overexpressed from multicopy plasmids pME6220 (*algU*⁺) and pME6217 (*algU*⁺*mucA*⁺) (Table 1; Fig. 1) in strain CHA0, the level of EPS production increased to the level observed in *mucA* mutant CHA213M (Table 2). Interestingly, the presence of multiple copies of *mucA* in CHA0 carrying pME6217 did not interfere with the EPS-stimulating effect of the *algU* amplification (Table 2), perhaps because other regulators, such as *MucB*, were not present at titers that were high enough. Constitutively high levels of expression of *algU* and *mucA* from the *lac* promoter on plasmid pME6555 (Table 1; Fig. 1) in strain CHA0 resulted in further increases in the level of EPS production to levels that were about 3.5 times greater than the level in *mucA* mutant CHA213M (Table 2). Attempts to clone *algU* alone under the control of the *lac* promoter failed, since *E. coli* and *P. fluorescens* transformants carrying plasmid constructs with such an insert were not viable and did not maintain the plasmid, respectively (unpublished data). Similar problems have been reported for attempts to overexpress *algU* in *P. aeruginosa* and were attributed to potential toxicity of *AlgU* in the absence of its negative regulators, *MucA* and *MucB* (22, 55). In conclusion, EPS biosynthesis in strain CHA0 is controlled by a fine-tuned balance between *AlgU* and *MucA*, probably assisted by *MucB*.

Biochemical analysis of EPS from mucoid mutants of *P. fluorescens*. EPS was extracted and purified from mucoid material of KMB agar cultures of *mucA* mutants CHA211 and CHA213M by using the procedure described by Pedersen et al. (47). During DEAE-Sephacrose ion-exchange chromatography, EPS of both *P. fluorescens* mutants, like alginate of *P. aeruginosa* (47), eluted between 0.4 and 0.7 M ammonium carbonate, with a maximum peak at 0.53 M ammonium carbonate. The *P. fluorescens* EPS preparations had total carbohydrate contents of 62% ± 9% and 87% ± 12% (on a dry weight basis) when seaweed alginate and D-mannuronic acid lactone, respectively, were used as the standards. When either of these standards was used, 46% ± 5% of the dry weight could be attributed to uronic acids, a value which is lower than the uronic acid content (70 to 100%) described for *P. aeruginosa* alginate (47). Nevertheless, the degree of acetylation of *P. fluorescens* uronic acids determined by a colorimetric assay was 16% ± 2%, a value identical to the value obtained for *P. aeruginosa* (47). Acetylation of uronic acid polymers is a typical feature of bacterial alginates, whereas algal alginates are not acetylated (18). No contaminating proteins or nucleic acids were detected in purified EPS from *P. fluorescens*. These results indicate that mucoidy in *mucA* mutants of *P. fluorescens* CHA0 is due to overproduction of an acetylated EPS which may be related to some extent to the EPS produced by *P. aeruginosa*. Fluorescent pseudomonads have been shown to produce a wide variety of EPS which may differ considerably in composition, and alginate has been detected in only some of these EPS (16, 17).

Kinetics of *algU* expression in response to osmotic stress. To test *algU* regulation in response to osmotic stress, we monitored expression of an *algU'*-*lacZ* reporter fusion carried by pME6222 (Table 1; Fig. 1) in wild-type strain CHA0 and mutant derivatives of this strain in the presence of NaCl and the nonionic solute sorbitol. In KMB without NaCl, *algU* was expressed at low, almost constant levels in strain CHA0 and in *algU* mutant CHA212 (Fig. 2A). Upon exposure to 0.6 M NaCl, *algU* expression in wild-type cells was transiently induced about two- to threefold in the early exponential growth phase, whereas no induction occurred in the *algU* mutant (Fig.

TABLE 3. Expression of an *algU*'-'*lacZ* fusion in *P. fluorescens* CHA0 and mutant derivatives of strain CHA0 in response to osmotic stress

Strain ^a	Genotype	β -Galactosidase activity (Miller units) ^b			Induction factor ^c	
		Without stress	0.6 M NaCl	1.2 M Sorbitol	NaCl	Sorbitol
CHA0	Wild type	174 b	448 b	469 b	2.6	2.7
CHA212	$\Delta algU$	104 c	77 c	124 b	0.7	1.2
CHA0/pME6555	P_{lac} - <i>algU</i> ⁺ <i>mucA</i> ⁺	275 a	683 a	657 a	2.5	2.4
CHA213M	$\Delta mucA$	268 a	694 a	ND	2.6	ND
CHA89	<i>gacA</i> :: Ω -Km	147 b	381 b	ND	2.6	ND
CHA510	<i>gacS</i> ::Tn5	151 b	379 b	ND	2.5	ND

^a All bacterial strains carried the reporter construct pME6222 containing an *algU*'-'*lacZ* fusion.

^b The initial bacterial cell concentration was adjusted to an OD₆₀₀ of 0.05, and cells were grown for 12 h at 30°C in KMB broth with no supplement or amended with 0.6 M NaCl or 1.2 M sorbitol. The data are means based on three experiments, and three replicate cultures were used for each experiment. Values in the same column followed by different letters are significantly different at *P* = 0.05 (Student's *t* test). ND, not determined.

^c Expression of *algU*'-'*lacZ* in a bacterial strain grown in the presence of a stress relative to expression of the reporter construct in the absence of the stress.

2B). Similar results were obtained when an osmotically equivalent concentration of sorbitol (1.2 M) was added to the growth medium (Table 3). Overexpression of *algU* from pME6555 or the lack of *mucA* in strain CHA213M led to expression of the *algU*'-'*lacZ* reporter that was significantly greater than the expression in the wild type in the nonsupplemented medium (Table 3). Expression of *algU*'-'*lacZ* was further enhanced by exposure to osmotic stress (Table 3). In summary, *algU* expression in *P. fluorescens* is induced in response to osmotic stress, and moreover, as in *P. aeruginosa* (12, 22, 56, 69), AlgU in *P. fluorescens* positively regulates its own expression.

In *P. fluorescens* CHA0 a conserved two-component regulatory system consisting of the sensor kinase GacS and the cognate response regulator GacA globally controls the biosynthesis of a range of extracellular products, including antimicrobial compounds and exoenzymes (20, 29). EPS is another exoproduct of strain CHA0 controlled by the GacS-GacA system, as disruption of the *gacA* gene in mucoid *mucA* mutant CHA213M.*gacA* made the strain nonmucoid and reduced the levels of EPS production to the levels in the wild type or *gacA* mutant CHA89 (Table 2). GacS-GacA also regulates alginate production in *P. syringae* (67), *Pseudomonas viridiflava* (30), and *A. vinelandii* (9). In *P. fluorescens*, *algU* expression in *gacA* or *gacS* mutants was not significantly different from *algU* expression in the wild type in the absence or presence of osmotic stress (Table 3), indicating that GacS-GacA control of EPS production is not exerted via the sigma factor AlgU.

AlgU is required for tolerance of *P. fluorescens* towards osmotic stress. We next tested how different levels of *algU* ex-

pression affect growth and survival of *P. fluorescens* in response to osmotic stress. To do this, the doubling times of strain CHA0 and mutant derivatives of this strain were determined during exponential growth of the bacteria in KMB supplemented with different concentrations of NaCl or sorbitol. The *algU* mutant CHA212 was significantly more sensitive to osmotic stress (0.6 or 0.8 M NaCl, 1.2 or 1.6 M sorbitol) than the wild type and the *algU* mutant complemented with pME6221 (Table 4). After 24 h of incubation, the numbers of CFU obtained from NaCl-stressed cultures of the *algU* mutant were about 10 to 30 times lower than the numbers of CFU obtained from cultures of the wild type and the complemented mutant exposed to the same stress (data not shown). Constitutive expression of *algU* from the *lac* promoter in CHA0/pME6555 made the strain hypersensitive to osmotic stress instead of protecting it (Table 4). Since high levels of AlgU may be toxic to bacterial cells (22, 55), this may have accounted for the increased sensitivity of strain CHA0/pME6555 under stress conditions. Mutant CHA213M lacking the anti-sigma factor MucA exhibited stress tolerance similar to that of the wild type (Table 4). Analogous results were obtained when bacterial strains were exposed to 1.0 M NaCl (data not shown). None of the strains tested was able to grow in medium supplemented with 1.2 M NaCl. In conclusion, AlgU is essential for adaptation of *P. fluorescens* to osmotic stress, but tolerance towards this stress apparently cannot be improved by overexpression of AlgU.

AlgU is essential for tolerance of *P. fluorescens* towards desiccation stress in vitro and in soil microcosms. To investigate whether AlgU contributes to tolerance of *P. fluorescens* to-

TABLE 4. Effect of osmotic stress on the growth of *P. fluorescens* CHA0 and derivatives of this strain

Strain ^a	Genotype	Doubling time (h) ^b				
		Without stress	0.6 M NaCl	0.8 M NaCl	1.2 M Sorbitol	1.6 M Sorbitol
CHA0	Wild type	0.72 a	1.63 c	3.75 c	1.88 b	3.05 b
CHA212	$\Delta algU$	0.72 a	2.01 b	7.83 a	2.82 a	NG ^c
CHA212/pME6221	$\Delta algU$ / <i>algU</i> ⁺	0.72 a	1.71 c	3.97 c	1.92 b	3.72 b
CHA0/pME6555	P_{lac} - <i>algU</i> ⁺ <i>mucA</i> ⁺	0.73 a	2.53 a	6.23 b	1.94 b	5.59 a
CHA213M	$\Delta mucA$	0.75 a	1.73 c	4.03 c	ND	ND
CHA213M/pME6219	$\Delta mucA$ / <i>mucA</i> ⁺	0.73 a	1.72 c	3.79 c	ND	ND

^a Bacteria were grown at 30°C in KMB broth with and without NaCl or sorbitol.

^b Doubling time was calculated from OD₆₀₀ values determined during the exponential growth phase (six to eight measurements). During this time the relationship of OD₆₀₀ values to CFU counts was linear (data not shown). The data are means based on three independent experiments, and three replicate cultures were used for each experiment. Values in the same column followed by different letters are significantly different at *P* = 0.05 (Student's *t* test). ND, not determined.

^c NG, no measurable growth during 48 h.

FIG. 3. Survival of *P. fluorescens*. Strain CHA0 (wild type) (●) and its derivatives CHA212 ($\Delta algU$) (○), CHA212/pME6010 ΔB ($\Delta algU/-$) (Δ), and CHA212/pME6221 ($\Delta algU/algU^+$) ($\blacktriangle$) were included in a desiccating sterile artificial soil consisting of a mixture of quartz sand and vermiculite (A) and in a desiccating natural sandy loam soil (B). For microcosms containing natural soil, rifampin-resistant derivatives of the bacterial strains were used as inoculants. The soil water content ($\diamond$) at different times is shown only for the treatment with wild-type strain CHA0 since it did not vary among the different treatments. In the artificial soil, water contents of 15, 10, 5, 1, and 0.5% corresponded to soil Ψ_w of about -0.01 , -0.03 , -0.5 , -2.7 , and -40 MPa, respectively. In the natural soil, water contents of 20, 15, 10, and 1% corresponded to Ψ_w of about -0.03 , -1.5 , -3.0 , and -9.5 MPa, respectively. The data are means $\pm$ standard deviations based on three experiments. Some of the error bars were too small to be included.

wards desiccation stress, strain CHA0 and *algU* and *mucA* mutants of this strain were exposed to desiccation stress under various conditions. In the first series of experiments, bacterial colonies grown on filter disks were slowly desiccated for 24 h or were kept at a constant humidity (see Materials and Methods). The numbers of CFU obtained from desiccated colonies of wild-type strain CHA0 were 7% of the numbers of CFU obtained from nondesiccated control colonies. The *algU* mutant CHA212 did not survive this treatment ($<0.1\%$ survival). Complementation of strain CHA212 with *algU*⁺ plasmid pME6221 restored survival to wild-type levels (9%). *mucA* mutant CHA213M had a wild-type level of survival (8%), suggesting that a lack of AlgU inhibition and enhanced EPS production do not improve survival in response to desiccation stress.

In a second series of experiments, the contribution of AlgU to the stress tolerance of *P. fluorescens* was examined in desiccating artificial and natural soil microcosms. The population dynamics of wild-type strain CHA0 and mutants of this strain were monitored for several weeks and were related to the decrease in the soil water content. In a sterile mixture of vermiculite and quartz sand that mimicked formulation conditions, the sizes of the populations of all bacterial strains started to decline at soil water contents below 5% (Fig. 3A), which corresponded to a soil Ψ_w of about -0.5 MPa. The decline in the size of the population was much more rapid for *algU* mutant CHA212, and the number of CFU per gram (dry weight) of soil was up to 4 log units lower than the value obtained for the wild type 10 days after bacteria were added to the soil (Fig. 3A). From day 15, no cultivable cells of the *algU* mutant were recovered from the soil (Fig. 3A). In contrast, wild-type strain CHA0 was detected for up to 60 days after inoculation (Fig. 3A; data not shown). Interestingly, strain CHA212 complemented with intact *algU* carried by pME6221 appeared to be slightly more tolerant towards desiccation stress than the wild type, whereas introduction of cloning vector pME6010 ΔB alone had no effect on the sensitivity to stress (Fig. 3A). No significant differences among the strains tested

were observed in control experiments in which the water content of the vermiculite-sand mixture was kept constant at 15%. Under these conditions, the bacterial population densities remained stable at about 0.6×10^7 to 1.1×10^7 CFU/g (dry weight) of soil throughout the 6-week experiment.

Similar results were obtained when strain CHA0 and *algU* mutant CHA212 were exposed to desiccation stress in a natural, nonsterile, sandy loam soil (Fig. 3B). For this experiment, the rifampin-resistant derivatives CHA0-Rif and CHA212-Rif (Table 1) were used. These derivatives were indistinguishable from their parent strains in terms of growth, EPS production, and tolerance towards osmotic stress and desiccation on filter disks (data not shown). The natural soil had a better water retention capacity than the artificial soil, which resulted in a lower desiccation rate and, consequently, in less rapid declines in the sizes of the bacterial populations (Fig. 3B). However, as soon as the soil water content dropped below a critical level, about 5% (i.e., a Ψ_w of about -4.9 MPa), the *algU* mutant again was significantly more sensitive to desiccation stress than the wild type or the complemented mutant (Fig. 3B). In natural soil kept at a constant moisture level, the sizes of the populations of all bacterial strains remained at equally high levels, and there was a slight decline from 10^7 to 10^6 CFU/g (dry weight) of soil until the end of the 30-day experiment. Taken together, these results demonstrate that the sigma factor AlgU is a crucial determinant in the adaptation of *P. fluorescens* to desiccation stress.

AlgU is not required for tolerance of *P. fluorescens* towards ROIs and heat. AlgU contributes to the tolerance of the pathogens *P. aeruginosa* and *P. syringae* towards certain agents that generate ROIs and towards heat (26, 32). To determine whether inactivation of *algU* or *mucA* affected the susceptibility of *P. fluorescens* to ROIs, wild-type strain CHA0, *algU* mutant CHA212, and *mucA* mutant CHA213M were exposed to 1.9% (wt/vol) paraquat, 3% (vol/vol) hydrogen peroxide, or 5% (vol/vol) sodium hypochlorite. Table 5 shows that both mutants displayed wild-type susceptibility to these agents. Additional experiments in which the concentrations of the

TABLE 5. Sensitivity to killing by ROIs in *algU* and *mucA* mutants of *P. fluorescens* and *P. aeruginosa*

Strain	Characteristics	Mean diam of growth inhibition zone (mm) with:		
		Paraquat (1.9%)	H ₂ O ₂ (3%)	NaOCl (5%)
<i>P. fluorescens</i> strains				
CHA0	Nonmucoid, wild type	18.0 a	12.0 a	6.5 a
CHA212	Nonmucoid, $\Delta algU$	17.0 a	12.0 a	6.5 a
CHA213M	Mucoid, $\Delta mucA$	19.0 a	12.5 a	6.5 a
<i>P. aeruginosa</i> strains				
PAO1	Nonmucoid, wild type	14.0 y	7.5 x	7.0 x
PAO6852	Nonmucoid, $algU::Tc^r$	22.0 x	9.5 x	8.5 x
PAO568	Mucoid, $mucA2$	14.0 y	9.0 x	8.0 x

^a Sensitivities to paraquat, H₂O₂, and NaOCl are expressed as the diameters of the growth inhibition zones surrounding filter disks impregnated with 10- μ l portions of the agents. The data are means based on three replicates. Values in the same column followed by different letters (a for *P. fluorescens*, x and y for *P. aeruginosa*) are significantly different at $P = 0.05$ (Student's *t* test).

ROI-generating agents were increased up to 10-fold gave analogous results (data not shown). In contrast, an *algU* mutant (PAO6852) of *P. aeruginosa*, which was included as a control in the same experiment, was significantly more sensitive to paraquat, but not to H₂O₂ and NaOCl, than wild-type strain PAO1 and mucoid strain PAO568 were (Table 5), confirming previous data of Martin et al. (32). A series of experiments was then performed to evaluate the role of AlgU in tolerance of *P. fluorescens* towards heat killing. Exponential-growth-phase cultures of strain CHA0 and *algU* mutant CHA212 were exposed to 42 or 48°C for 5 to 90 min. However, in none of the experiments was there a significant difference in viability between the wild type and the *algU* mutant (data not shown). In an experiment in which we screened for additional factors that may involve the AlgU-mediated stress response, we found no differences in the tolerance of strain CHA0 and its *algU* mutant towards a series of other stresses, including exposure to the strong reducing agent dithiothreitol (8 mM), which triggers RpoE function in *E. coli* (44), exposure to a range of antibiotics, and exposure to pH extremes (data not shown). In summary, these results suggest that AlgU is not required for tolerance towards ROIs and heat in *P. fluorescens*, in marked contrast to the role of this sigma factor in *P. aeruginosa*, *P. syringae*, and *E. coli*.

DISCUSSION

In the present study, we identified the *algU-mucA-mucB* gene cluster in the plant-beneficial strain *P. fluorescens* CHA0; this gene arrangement is conserved in *P. aeruginosa* (12, 31, 33) and *P. syringae* (26). In *P. aeruginosa*, the *algU*, *mucA*, and *mucB* genes are cotranscribed and encode (respectively) the sigma factor AlgU and its main negative regulators, MucA and MucB, which act in concert to control production of the EPS alginate and the response to extreme environmental stress (19, 32, 50, 54, 57, 70). In *P. syringae*, AlgU is also a major determinant in the regulation of alginate biosynthesis and stress response (26). Here, we provide evidence that on the one hand, AlgU of *P. fluorescens* is functionally equivalent to its counterparts in pathogenic pseudomonads with respect to control of EPS production. On the other hand, the role of AlgU in

the stress response of *P. fluorescens* is distinct from its role in the stress responses of other bacteria.

Regulation of EPS production. In *P. fluorescens* CHA0, EPS production was regulated by at least four mechanisms. First, the level of *algU* expression was important. This was seen most clearly in a strain with moderate *algU* overexpression, CHA0/pME6220, which overproduced EPS (Table 2). The level of *algU* expression is determined by AlgU itself: expression of an *algU'*-*lacZ* reporter was about 40% lower in an *algU* mutant than in wild-type strain CHA0 (Fig. 2A; Table 3). AlgU auto-regulation has also been described for *P. aeruginosa* (12, 22). The *algU* promoter region in *P. fluorescens* CHA0 was found to contain two putative AlgU/RpoE recognition sequences. In *P. aeruginosa*, the corresponding promoters are designated P1 and P3, and they are absolutely dependent on AlgU (56). Second, the anti-sigma factor MucA made a major contribution to EPS production. Without MucA the EPS levels were 100-fold higher than those in a MucA⁺ background (Table 2). In contrast, the (indirect) effect of MucA on *algU* transcription was relatively small (Table 3). Third, the GacS-GacA two-component system was essential to sustain high EPS productivity in a *mucA* mutant (Table 2). This positive effect of the transcriptional regulator GacA was not mediated by AlgU (Table 3). Fourth, the hypermucoid phenotype of strains CHA211 (*mucA*::Tn5) and CHA0/pME6555 (P_{lac}-*algU*⁺*mucA*⁺) points to negative AlgU control by MucB, as in *P. aeruginosa* (36, 50).

Role of AlgU in tolerance towards environmental stress. Little is known about the regulatory mechanisms that determine adaptation of plant-beneficial pseudomonads to environmental stress. The present study established that AlgU is a second sigma factor, in addition to RpoS (52), involved in the response of *P. fluorescens* to extreme environmental stress. Our work shows, for the first time, that AlgU is a key determinant in the tolerance of *P. fluorescens* towards desiccation stress, since the survival of an *algU* mutant was severely impaired when the organism was exposed to desiccation in vitro, in a vermiculite-sand mixture mimicking formulation conditions, and in natural soil (Fig. 3). Furthermore, this sigma factor was important in the adaptation of *P. fluorescens* to high-osmolarity conditions (Table 4), and high concentrations of NaCl or sorbitol stimulated *algU* expression in both *P. fluorescens* (Table 3; Fig. 2) and *P. syringae* (26). Since plant-associated pseudomonads may be exposed to dry and osmotically harsh conditions in the rhizosphere and in the phyllosphere, the capacity to adapt to these conditions is likely to be important for colonization of these plant surfaces (26, 42, 63).

High osmolarity and ethanol (as a dehydrating agent) enhance transcription of alginate biosynthesis genes in *P. aeruginosa* (1, 11, 14, 71) and *P. syringae* (48). Osmotic stress and dehydration stress also stimulate, to a limited extent, alginate production in *P. syringae* and certain *P. fluorescens* strains (49, 58). However, exposure of nonmucoid *P. aeruginosa* (1, 56) or *P. fluorescens* CHA0 (unpublished data) to osmotic stress does not fully induce EPS synthesis, indicating that other environmental factors and cellular regulators are required for full activation of the EPS biosynthesis genes. Remarkably, the highly mucoid, *algU*-overexpressing or *mucA* mutants of *P. fluorescens* CHA0 did not display improved tolerance towards osmotic stress (Table 4) and desiccation in vitro and in soil microcosms (Table 5; unpublished data). This might argue

against a protective role for EPS in *P. fluorescens* CHA0. Nevertheless, we cannot rule out the possibility that the soil conditions used were not conducive to enhanced EPS production by the mucoid variants. In this context it is noteworthy that mucoid mutants of *P. fluorescens* CHA0 have exhibited an improved ability to form biofilms on roots (2). However, it is also possible that the (low) wild-type level of EPS production was already sufficient for maximum EPS-mediated stress protection of the bacterium and that stress defense may not be further optimized by EPS overproduction.

At present, it is difficult to explain the fact that the *algU*-overexpressing mutants (i.e., CHA213M and CHA0/pME6555) (Table 3) failed to be more stress tolerant than wild-type strain CHA0, as we expected these mutants to overexpress defense-related functions in addition to EPS overproduction. One possibility is that AlgU acts as an on-off switch which, when activated, drives the expression of stress defense-related genes. This system may operate until a certain level of saturation of available active AlgU is reached; beyond this level, AlgU may even become toxic to the cell.

Interestingly, an *algU* mutant of *P. fluorescens* CHA0 was not hypersensitive to a high temperature (48°C) (data not shown) or paraquat, H₂O₂, and NaClO (Table 5). This contrasts with the demonstrated involvement of AlgU in tolerance towards these stresses in *P. aeruginosa* (32, 54, 56, 70) and in *P. syringae* (26). Activation of *algU* by ROIs may help these pathogens withstand the oxidative burst associated with host defense responses (19, 26). This AlgU-mediated mechanism may not be required in the nonpathogenic strain *P. fluorescens* CHA0, as this bacterium may not be critically exposed to the plant defense response. Alternatively, the sigma factor RpoS, which is required for tolerance towards oxidative stress in the closely related plant-beneficial bacterium *P. fluorescens* Pf-5 (52), might ensure protection of strain CHA0 from plant ROIs.

ACKNOWLEDGMENTS

We gratefully acknowledge financial support from the Swiss Federal Office for Education and Science (project C99.0032, COST action 830) and the Swiss Priority Program Biotechnology (project 5002-04502311).

We thank Patrick Michaux for help with some of the experiments. We are grateful to Stephan Heeb, Cécile Gigot-Bonnefoy, and Cornelia Reimann for advice and to Fabio Mascher for help with determining the soil ψ_w .

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