JOURNAL OF BACTERIOLOGY, Jan. 1997, p. 187–193 Vol. 179, No. 1
0021-9193/97/$04.00+0

Identification of the *algZ* Gene Upstream of the Response Regulator *algR* and Its Participation in Control of Alginate Production in *Pseudomonas aeruginosa*

H. YU, M. MUDD, J. C. BOUCHER, M. J. SCHURR, AND V. DERETIC*

Department of Microbiology and Immunology, The University of Michigan Medical School, Ann Arbor, Michigan 48109-0620

Received 6 September 1996/Accepted 21 October 1996

Alginate production in mucoid *Pseudomonas aeruginosa* isolates from cystic fibrosis patients is under direct control by AlgU, the *P. aeruginosa* equivalent of the extreme heat shock sigma factor σ^E in gram-negative bacteria, and AlgR, a response regulator from the superfamily of two-component signal transduction systems. In this report, we describe the identification of the *algZ* gene, located immediately upstream of *algR*, which is involved in the control of alginate production. The predicted product of the *algZ* gene showed similarity to a subset of sensory components from the superfamily of signal transduction systems but lacked several of the highly conserved motifs typical of histidine protein kinases. Inactivation of *algZ* in the wild-type standard genetic strain PAO1 did not affect its nonmucoid morphology. However, inactivation of *algZ* in a mucoid mutant *P. aeruginosa* strain, which had AlgU freed from control by the anti-sigma factor MucA, resulted in increased alginate production under growth conditions which did not permit expression of mucoidy in the parental *algZ*$^+$ strain. The observed effects were abrogated when *algR* was inactivated in the *algZ*::Tc^r background. These findings indicate that *algZ* plays a regulatory role in alginate production, possibly interacting with AlgR, and that it may have negative effects on expression of the mucoid phenotype under the conditions tested. The presented results suggest that elements of negative regulation exist at the levels of both the alternative sigma factor AlgU and the transcriptional activator AlgR which, once relieved from that suppression, cooperate to bring about the expression of the alginate system.

Conversion of *Pseudomonas aeruginosa* to the mucoid, exopolysaccharide alginate-overproducing phenotype is usually associated with chronic colonization of the respiratory tract in cystic fibrosis (18). Recent analyses have shown that the conversion to mucoidy in cystic fibrosis isolates is caused by mutations in the *mucA* gene (5, 30, 48) located within the *algU mucABCD* cluster at 67.5 min (5, 28, 29). AlgU (also known as AlgT [13, 16] or Pa σ^E [11]) is a member of the AlgU-RpoE subset of alternative σ factors from the superfamily of σ^E-like (12) or ECF (27) sigma subunits of RNA polymerase. In wild-type nonmucoid *P. aeruginosa*, AlgU is inhibited by the anti-σ factor MucA (42, 46). Inactivation of *mucA* relieves AlgU from repression and causes conversion to mucoidy (5, 30, 42).

AlgU (Pa σ^E) directs transcription of promoters characterized by the σ^E promoter consensus sequence −35 GAACTT ($N_{16/17}$) −10 TCTgA (11, 15, 26, 31, 40). At least two genes critical for alginate production, *algD* and *algR*, have σ^E promoters and depend on AlgU for transcription (11, 31). The *algD* gene encodes a key alginate biosynthetic enzyme, GDP-mannose dehydrogenase (32), and is located at the beginning of the gene cluster at 34 min which encodes alginate biosynthetic and modification enzymes (32). The *algR* gene is located at 9 min of the *P. aeruginosa* chromosomal map (Fig. 1) and encodes a transcriptional activator which binds to the *algD* promoter (7, 20, 34, 35). AlgR, most likely in concert with the alternative sigma factor AlgU, induces *algD* transcription. AlgR is a member of the superfamily of bacterial two-component systems and is homologous to response regulators (7). Two-component signal transduction systems typically consist of at least two factors, the response regulator and its cognate sensor (44). The response regulators have highly conserved N-terminal receiver domains which undergo phosphorylation in the process of signal transduction (38, 44). The sensory component usually acts as a histidine protein kinase and serves as a source of phosphoryl groups for the cognate response regulator but sometimes also accelerates its dephosphorylation (44). While AlgR has a typical N-terminal receiver domain and can undergo in vitro phosphorylation in interactions with heterologous protein kinases and low-molecular-weight phosphoryl group donors (10), the putative second component interacting with AlgR was not identified.

Here we report the identification of *algZ*, a gene located immediately upstream of *algR*. The predicted product of the *algZ* gene is homologous to a narrow subset of putative sensors that have been recently classified in the two-component regulatory systems (6). We also show that AlgZ plays a negative regulatory role in alginate production with phenotypically appreciable effects in a mucoid *mucA* mutant background.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *P. aeruginosa* strains and plasmids used in this study are shown in Table 1. PAO6886 was the *algZ*::Tc^r derivative of PAO1. PAO6884 and PAO6885 were *algZ*::Tc^r and *algZ*::Tc^r *algR*::Gm^r derivatives of PAO568, respectively. *P. aeruginosa* was grown on *Pseudomonas* isolation agar (PIA; Difco) or LB supplemented with tetracycline (300 or 50 μg/ml for PIA or LB, respectively) or gentamicin (150 or 30 μg/ml) when required. *Escherichia coli* was grown on LB supplemented with ampicillin (100 μg/ml), kanamycin (25 μg/ml), or gentamicin (13 μg/ml) when required. Bacterial growth curves were determined as previously described (5). For extraction of RNA and total cellular proteins for immunoblotting analysis, bacteria were grown in LB to an optical density at 600 nm of 0.4 at 37°C before rapid cooling in a dry ice-ethanol bath to 4°C and harvesting for cell lysis. For quantitation of alginate production, bacteria were grown on low-osmolarity medium

* Corresponding author. Mailing address: Department of Microbiology and Immunology, The University of Michigan Medical School, 5641 Medical Science Building II, Ann Arbor, MI 48109-0620. Phone: (313) 763-1580. Fax: (313) 647-6243. E-mail: Deretic@umich.edu.

FIG. 1. Genetic organization of the *algZR* locus and its vicinity. The *algZ* and *algR* genes participate in the control of alginate production in *P. aeruginosa*. AlgR is a transcriptional activator which binds to *algD* (20, 34, 35) as a necessary step in the activation of alginate production. AlgZ (GenBank accession number U50713) negatively regulates alginate production in an AlgR-dependent fashion and may act as a second component of this signal transduction system. Upstream of *algZ* is the *argH* gene involved in the biosynthesis of arginine (33). Downstream of *algR* are the *hemC* and *hemD* genes involved in the biosynthesis of heme precursors (37). Linked, but not immediately adjacent, to these genes are *algQ* (*algR2*), *fkl*, and *algP* (*algR3*). The *fkl* gene (GenBank accession number M32077) encodes a homolog of FK506-binding proteins from the immunofilin superfamily. AlgQ and AlgP have been implicated in the control of alginate production (21, 22). AlgQ is a homolog of PfrA, a siderophore regulator for *Pseudomonas putida* (45), while AlgP is a putative histonelike element (20).

(per liter of H_2O, we added 10 g of peptone, 5 g of yeast extract, 3 g of NaCl, and 15 g of agar, pH 7.0) at 37°C for 48 h.

Genetic methods. Gene replacements and insertional inactivation of chromosomal genes were carried out as previously described (29). The *algR* and *algZ* genes were insertionally inactivated on the *P. aeruginosa* chromosome via homologous recombination with nonreplicative plasmids pKRG1 and pSSZ1, respectively. To generate pSSZ1, a 1.59-kb *Eco*RI-*Not*I fragment carrying *algZ* was digested with *Pst*I and a 58-bp *Pst*I fragment internal to *algZ* (see Fig. 3) was replaced with the Tc^r cassette (1.4 kb) from pKI11. The *algZ*::Tc^r fragment was cloned into pBluescript SKII (Stratagene), and a *Hin*dIII fragment with *mob* from pCMobA was added to generate pSSZ1. To construct pKRG1, the *Bgl*II fragment (6 kb) with *algR* in pT7-6 was digested with *Nru*I (351 bp downstream from ATG of *algR*) and this site was modified with *Bam*HI linkers to permit insertion of the *Bam*HI Gm^r cassette (1.6 kb) from pKI11G. The *mob* function was transferred as a *Hin*dIII fragment from pCMobA to this plasmid to generate pKRG1. pSSZ1 and pKRG1 were introduced into the recipient strains by triparental conjugations. Candidate strains with gene replacements done via double-crossover events between a plasmid and the chromosome were isolated as Gm^r Cb^s or Tc^r Cb^s exconjugants selected by replica plating. All gene replacements were confirmed by Southern blot analysis.

DNA sequencing. For DNA sequencing of *algZ*, genomic DNA between *argH* (33) and *algR* (7) from two strains (PAO1 and PAO568) was amplified by PCR with oligonucleotides H4 (5′TTGGTCTTCTCTACGC3′) and R1 (5′GGTTCG TCATCGACAA3′). DNA sequencing of the 1.3-kb PCR product with *algZ* was carried out with an AmpliTaq Cycle Sequencing Kit (Perkin Elmer Cetus) and custom-made primers.

RNA isolation and S1 nuclease protection analysis. RNA isolation and S1 nuclease protection analysis were carried out as previously described (31, 40). Briefly, for RNA isolation, *P. aeruginosa* cells were washed twice in ice-cold 50 mM Tris (pH 7.5) and lysed in 3.3% sodium dodecyl sulfate (SDS)–50 mM Tris (pH 7.5), and total RNA was isolated by equilibrium centrifugation through a cushion of 5.7 M CsCl. S1 nuclease protection analysis was performed with a single-stranded DNA probe uniformly labeled with [α-^{32}P]dCTP (800 Ci/mmol; DuPont NEN) by using primer R1 (5′GGTTCGTCATCGACAA3′) and purified single-stranded M13 DNA containing the *algR* upstream region (Δ3/28) as the template. Following polymerization, the radiolabeled probe was digested with *Xho*I, heat denatured in formamide, gel purified, and annealed to 50 μg of total RNA for 1 h at 67°C. After annealing, the DNA-RNA hybrid was digested for 30 min at 37°C by addition of 1,200 U of S1 nuclease (Boehringer Mannheim) and 300 μl of ice-cold S1 buffer containing 280 mM NaCl, 50 mM sodium acetate (pH 4.6), 4.5 mM ZnSO4, and 20 μg of denatured salmon sperm DNA per ml. S1 nuclease digestion products were precipitated with ethanol and analyzed on sequencing gels along with the sequencing ladder produced by using the same template and primer that were used to prepare the S1 nuclease probe.

Detection of the *algZ* gene product in a T7 expression system. For visualization of AlgZ in an *E. coli* T7 expression system, the 1.3-kb PCR product with *algZ* was first cloned into pCRII (Invitrogen), the DNA sequence of the cloned fragment was verified, and the 1.3-kb fragment was transferred as an *Eco*RI fragment into pT7-5 in both orientations. AlgZ was labeled with Expre^{35}S^{35}S protein mix (1,000 Ci/mmol; DuPont NEN) and visualized by using previously described methods (49).

Western blot analysis. *P. aeruginosa* cells were washed twice in ice-cold phosphate-buffered saline (10 mM, pH 7.4) and resuspended in 300 μl of phosphate-buffered saline in the presence of 50 μl of zirconium beads (0.1-mm diameter; Biospec Products). Cells were homogenized in a Mini Beadbeater for 3 min at 2,800 rpm. The cell debris and beads were removed by centrifugation. The protein concentration of the resulting supernatant was assayed, and the supernatant was mixed with an equal volume of 2× SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and analyzed on an SDS–11% PAGE gel as previously described (34). Proteins on the SDS-PAGE gel were transferred to Immobilon P membrane (Millipore) by electroblotting for immunodetection as described previously (10). A mouse monoclonal antibody against AlgR (10) was used in a dilution of 1:5,000 to probe AlgR. Goat anti-mouse immunoglobulin G (heavy and light chains) conjugated with horseradish peroxidase (Pierce) diluted 1:5,000 was used as the secondary antibody. The membrane was developed with H_2O_2 and diaminobenzidine as described previously (10).

Alginate assay. Production of alginate was determined by assaying uronic acids in a Shimadzu UV1601 spectrophotometer as previously described (23).

Nucleotide sequence accession number. The sequence of *algZ* from PAO1 (see Fig. 3) has been assigned GenBank accession number U50713.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant genotype or properties[a]	Source or reference
P. aeruginosa strains		
PAO1	Prototroph Alg^{-wt}	B. Holloway
PAO6886	PAO1 *algZ*::Tc^r Alg$^-$	This work
PAO568	Alg^{+m} *mucA2*	17
PAO6884	PAO568 *algZ*::Tc^r Alg^{+c}	This work
PAO6885	PAO568 *algZ*::Tc^r *algR*::Gm^r Alg$^-$	This work
Plasmids		
pKI11	pUC18 1.4-kb Tc^r cassette	S. Lory
pKI11G	pUC18 Gm^r cassette	S. Lory
pT7-5	ColE1 ϕ10 promoter; Ap^r	S. Tabor
pT7*algZ*	pT7-5 *algZ*$^+$	This work
pCMobA	pHC79 *mob*$^+$	33
pKRG1	pT7-6 *mob*$^+$ *algR*::Gm^r	This work
pSSZ1	pBluescript SKII *mob*$^+$ *algZ*::Tc^r	This work

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

RESULTS AND DISCUSSION

To identify the gene encoding the putative *algR*-cognate sensor, we have carried out mutational analysis of the chromosomal region in the vicinity of *algR* (33) following the rationale that the genes encoding cognate response regulators and sensors are typically closely linked (44). By using random mutagenesis strategies (33), two genes, *hemC* and *hemD*, have been identified immediately downstream of *algR* (Fig. 1) (37). Inactivation of a gene located 1.3 kb upstream of *algR* results in arginine auxotrophy, consistent with the sequence analysis that indicates the presence of the *argH* gene in the affected region (33). These experiments have left a segment of 1,306 bp between *argH* and *algR* that could accommodate an additional gene, suggesting the possibility that the *algR*-cognate sensor is in this region. To investigate this hypothesis, we carried out expression and sequence analyses of this region. Our T7 expression analysis indicated the presence of a gene upstream of *algR* encoding a polypeptide with an apparent molecular mass of 35 kDa (Fig. 2). DNA sequence analysis of the corresponding *argH-algR* intergenic region (Fig. 3) revealed that this gene, termed *algZ*, encodes a product displaying significant similarities (Fig. 4) to LytS (6) and YehU (GenBank accession number U00007), two recently reported sensors from the two-component systems. The smallest sum probability values [*P* (n)] assigned by the BLAST program (2) were 5.2×10^{-18} and 4.7×10^{-19}, respectively. These results are typical of those obtained within families of related proteins. AlgZ and YehU were 29% identical and 54% similar in an overlap of 359 amino acids. AlgZ and LytS were 24% identical (52% overall similarity) within the same region. LytS is a putative sensor proposed to interact with the response regulator LytR and controls the activity of murein hydrolase and the rate of autolysis

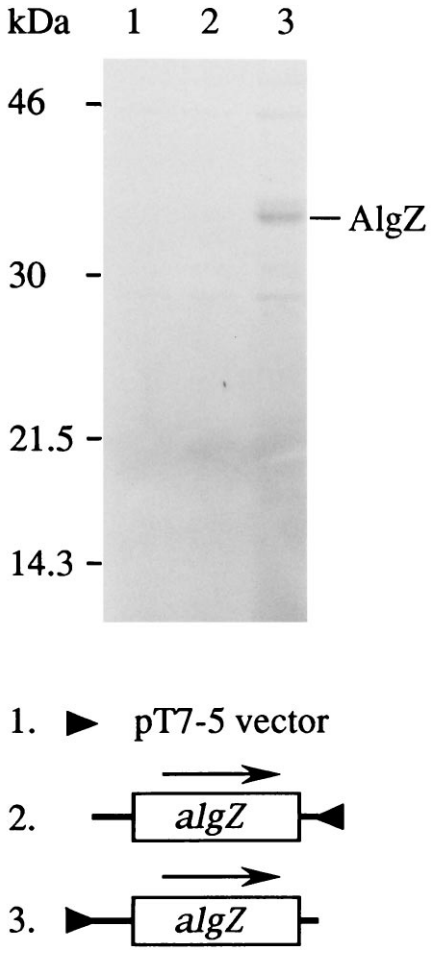

FIG. 2. Detection of the *algZ* gene product by T7 expression analysis. The *algZ* gene was cloned on a 1.3-kb fragment behind the T7 promoter in both orientations, and [^{35}S]Met- and [^{35}S]Cys-radiolabeled products were analyzed by SDS-PAGE and autoradiography. At the top is an autoradiogram of a gel with radiolabeled AlgZ. At the bottom is a schematic representation of the constructs used in T7 expression analysis. Lanes and constructs: 1, vector pT7-5; 2, pT7-5 containing the *algZ* gene in the direction opposite to T7 promoter transcription; 3, pT7-5 with the *algZ* gene inserted in the direction of T7 transcription. Triangle, T7 promoter.

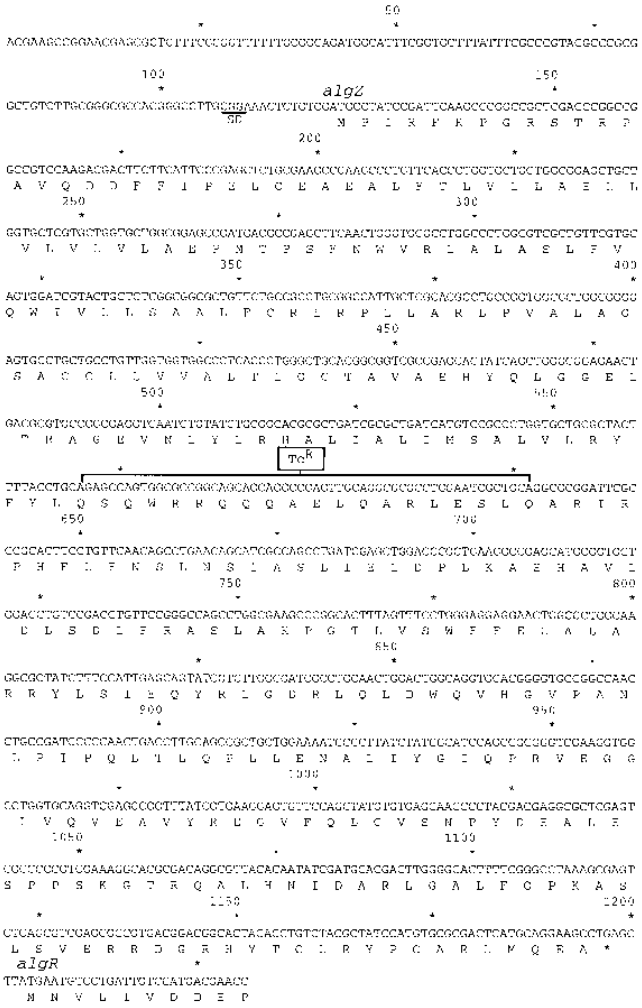

FIG. 3. Nucleotide sequence of the *P. aeruginosa algZ* gene. The 5′ end of the *algR* gene located immediately downstream of *algZ* is also displayed. Shown is the position of the Tc^r cassette (1.4 kb) replacing the 57-bp *Pst*I fragment within the *algZ* gene (overlined region) in *algZ* mutants. SD, putative Shine-Dalgarno sequence (ribosomal binding site).

in *Staphylococcus aureus* (6). The *yehU* gene is linked to a gene, *yehT*, encoding a putative response regulator (GenBank accession number U00007) in an arrangement reminiscent of two-component systems. These similarities suggest that *algZ* may encode the corresponding AlgR-cognate component.

To investigate whether *algZ* has any effects on alginate synthesis in *P. aeruginosa*, this gene was inactivated in the standard genetic wild-type nonmucoid strain PAO1. Only a minor, but nevertheless statistically significant, effect on the amounts of detectable uronic acids produced was observed (Table 2) in the *algZ*::Tc^r derivative of PAO1 (strain PAO6886). Somewhat surprisingly, the observed effect was an increase in alginate production (Table 2). Since the positive regulatory effect of *algR* on alginate production becomes appreciable only upon inactivation of this gene in mucoid *muc* mutants, it was of interest to investigate the effects of *algZ* inactivation in a *muc* background. Strain PAO568 (17) carries the previously characterized *mucA2* mutation (30) and has been used as a model strain to study control of mucoidy in *P. aeruginosa* (12, 18). The role of *algR* in alginate production in this strain has been well characterized (8, 36). PAO568 also has the useful property of being nonmucoid on low-osmolarity media but becomes mucoid as osmolarity increases (8, 33, 37). This allows examination of effects on alginate production under inducing or noninducing conditions. Inactivation of *algZ* in PAO568 did not significantly affect the phenotype of this strain on PIA, the medium on which PAO568 is fully mucoid. In contrast, loss of *algZ* caused significant enhancement of alginate production on low-osmolarity medium (Table 2). While the parental strain PAO568 (*mucA2 algZ*$^+$) was nonmucoid on this medium, strain PAO6884 (*mucA2 algZ*::Tc^r) displayed the mucoid phenotype on low-osmolarity medium (Fig. 5). These observations are consistent with the interpretation that *algZ*, unlike *algR*, which acts as a positive regulator, has a negative regulatory effect on alginate production in the *mucA* background tested.

The apparent negative effect of *algZ* could not be attributed to potential polar effects of *algZ* mutation on *algR* based on the following considerations and experiments. First, inactivation of *algR* abrogates alginate production, so any polar effects causing lower *algR* expression would be expected to reduce alginate synthesis. Second, the strong AlgU-dependent promoter of

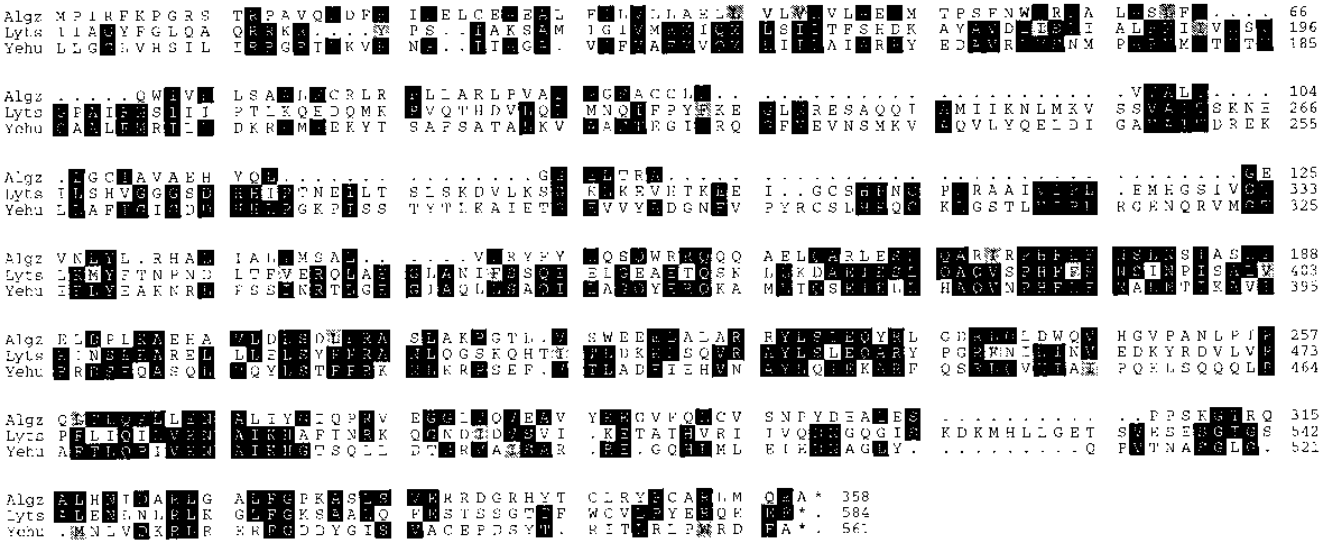

FIG. 4. Multiple sequence alignment of the predicted gene product AlgZ with LytS from *S. aureus* (6) and YehU from *E. coli* (GenBank accession number U00007). Only the regions displaying homologies are shown (133 to 584 for LytS, 119 to 561 for YehU, and the complete predicted sequence of AlgZ).

algR (9) is located downstream of the point of insertion of the Tc^r cassette in *algZ* and thus was expected to allow *algR* expression in PAO6884. This was experimentally verified by S1 nuclease protection analysis (Fig. 6A). Third, Western blot analyses with a monoclonal antibody against AlgR (10) revealed equal protein levels in both PAO568 (*algZ*$^+$) and PAO6884 (*algZ*::Tc^r) (Fig. 6B), confirming that AlgR levels remained unaltered in the *algZ* mutant. It is also worth mentioning that the Tc^r cassette on the chromosome of PAO6884 inserted in the orientation opposite to the transcription of the *algZ* and *algR* genes. The possibility that the observed phenotypic effect of the inactivation of *algZ* was due to the altered growth rate of the mutant was also eliminated. The *algZ*::Tc^r cells had the same growth characteristics as the parental strain when tested in the same medium. The doubling time of both PAO1 and PAO6886 was 33 min, while PAO568 and PAO6884 both had a doubling time of 29 min during the exponential phase of growth.

In further experiments, we investigated whether *algZ* exerted its effects via *algR*. The effects of *algZ* inactivation on the mucoid phenotype depended on *algR*, since inactivation of *algR* in *mucA2 algZ*::Tc^r strain PAO6884 abrogated alginate production (Table 2, strain PAO6885). Based on these analyses, it is possible to conclude that *algZ*, a gene tightly linked to *algR*, affects alginate production in an *algR*-dependent manner. Taken together with the sequence similarities to LytS and YehU, these data support AlgZ as a candidate for the second component interacting with the response regulator AlgR.

TABLE 2. Inactivation of *algZ* in *mucA P. aeruginosa* increases alginate production

Strain	Genotype	Phenotype[a]	Mean alginate production (μg/mg [wet wt] of cells) ± SE[b]
PAO1	Prototroph	NM	0.78 ± 0.21
PAO6886	PAO1 *algZ*::Tc^r	NM	1.20 ± 0.10
PAO568	*mucA2*	NM	1.87 ± 0.04
PAO6884	PAO568 *algZ*::Tc^r	M*	4.14 ± 0.14
PAO6885	PAO6884 *algR*::Gm^r	NM	0.91 ± 0.03

[a] Phenotypes of PAO568 and PAO6884 on LB plates: NM, nonmucoid; M*, mucoid after 48 h. All incubations were done at 37°C.

[b] The *P* values (*t* test) were 1.36×10^{-4} for PAO568 and PAO6884 and <0.05 for PAO1 and PAO6886.

Several features of the predicted primary structure of AlgZ are worth noting. First, sensory components frequently have transmembrane domains. Two segments of AlgZ that conform to the rules for possible transmembrane domains were detected (high hydrophobicity index [24] of regions with ≥20 amino acids [14]) within the N-terminal half of AlgZ: RLALA SLFVQWIVLLSAALFCR (residues 58 to 79; the underlined amino acids are charged residues demarcating the hydrophobic region) and RLPVALAGSACCLLVVALTLGCTAVAE (residues 86 to 112). Second, one intriguing feature of AlgZ is that it does not show similarities to the majority of other sensors sufficiently high to permit detection in global homology searches. Sequence analysis of the region upstream of *algR* did not show any significant similarities to proteins in the databases until now. Only upon the recent entry of LytS (6) and YehU (GenBank accession number U00007) was it possible to observe significantly strong homologies of AlgZ with any of the sequences deposited in databases. Some of the atypical features of the primary structure of AlgZ which deviate from the rest of the sensory components may help explain this paradox. Upon close inspection of its predicted sequence, it becomes apparent that AlgZ lacks complete motifs believed to be critical for the binding of ATP (38, 44). For example, although AlgZ has the equivalent of the N box, it does not have recognizable D/F and G boxes implicated in ATP binding (44). It is interesting that the other two close homologs of AlgZ, LytS and YehU, also lack fully developed corresponding motifs, albeit both LytS and YehU have two of three properly spaced Gly residues in the putative G region considered to be of importance for ATP binding. In contrast, AlgZ has only one Gly in the corresponding segment. However, several new regions of conservation are apparent in the alignments of AlgZ, LytS, and YehU (Fig. 4), suggestive of a distinct subfamily of sensors. For example, the strong conservation around the third

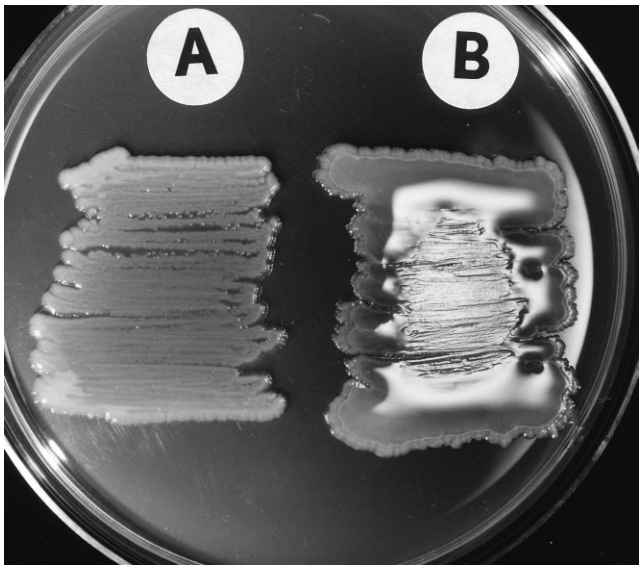

FIG. 5. Inactivation of *algZ* increases alginate production in an *mucA2* background. Shown is the morphology of strains PAO568 (*algZ*$^+$) (A) and PAO6884 (*algZ*::Tc^r) (B) grown on a subisotonic medium for 48 h at 37°C as described in Materials and Methods. Quantitation of the alginate production of these strains is given in Table 2.

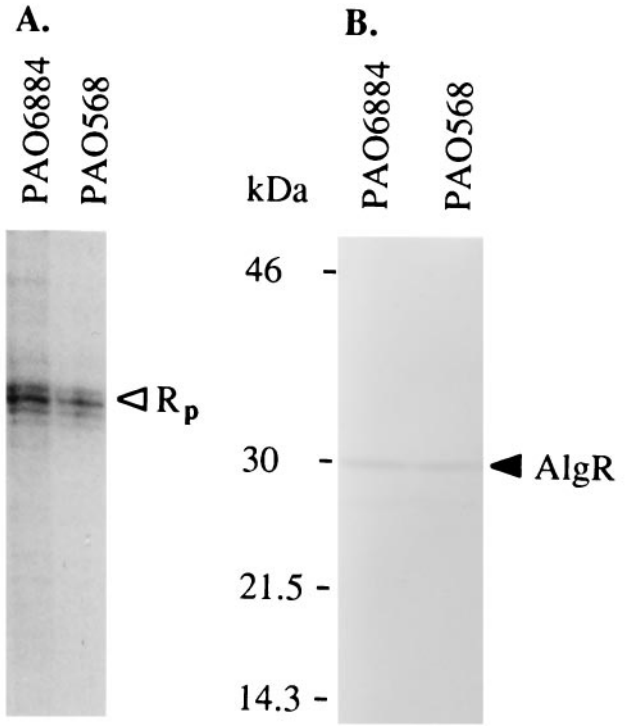

FIG. 6. Analysis of *algR* expression in an *algZ* mutant. (A) S1 nuclease protection analysis of the *algR* promoter. Fifty micrograms of the total RNAs of PAO568 and PAO6884 were hybridized with a uniformly radiolabeled *algR* probe, hybridization products were digested with S1 nuclease, and the resulting fragments were separated on a sequencing gel as described in Materials and Methods. R_p, *algR* proximal promoter. (B) Western blot analysis of AlgR levels in PAO568 (*algZ*$^+$) and its derivative PAO6884 (*algZ*::Tc^r). Twenty micrograms of the total cellular proteins of PAO568 and PAO6884 were separated by SDS–11% PAGE and, after transfer by electroblotting to an Immobilon P membrane, probed with monoclonal antibody 3H9 against AlgR (10) as described in Materials and Methods.

His residue of AlgZ (His-175) could potentially represent the phosphorylation site. This motif, LqAqvXPHFlFNslN (His-175 is underlined; capital letters are residues present in AlgZ, LytS, and YehU; lowercase letters are residues present in two of three sequences), is located 92 amino acids from the putative N box in AlgZ, matching the typical distance between the phosphorylated His residue and the N box in the majority of bona fide sensors (38, 44).

The implications of the lack of recognizable nucleotide-binding motifs in AlgZ are not clear. Further experiments are needed to assess whether AlgZ has kinase activity. In this context, it is also worth noting that the only phenotype associated with *algZ* inactivation is increased alginate production, although it is possible that under certain growth conditions *algZ* could play a positive regulatory role. The observed negative effects of *algZ* may potentially be explained by the phenomenon of sensor-dependent enhancement of phosphatase activity in the cognate response regulator. If phosphorylation of AlgR (e.g., via interaction with a kinase or low-M_r phosphodonors [10]) has a positive effect on alginate production, a supposition that remains to be explored, then AlgZ could act in a negative regulatory fashion by increasing the dephosphorylation rate of AlgR. There are known examples of sensors acting to accelerate the spontaneous rate of dephosphorylation of their cognate response regulators (1, 4, 19, 47). It is worth noting that even when the D/F and G boxes are removed from NR_{II} (NtrB), a situation that mimics the apparent absence of such domains in AlgZ, NR_{II} retains its phosphatase enhancement action (4). Although these considerations have no experimental support in the AlgZ-AlgR system, they provide a useful working model compatible with the sequence features of AlgZ and the observed phenotypic effects of *algZ* inactivation.

It may appear unusual that there are multiple regulatory factors controlling alginate production in *P. aeruginosa*. The existence of a multitiered negative regulation of alginate production at both the σ factor and response regulator levels may reflect the physiologically relevant phenomena associated with the conversion to mucoidy in *P. aeruginosa* and with bacterial response to extreme stress in general. First, it is possible that different factors specialize for different environmental inputs, which may increase the versatility of the system. Second, if AlgU is unchecked and overexpressed in the absence of at least one of its negative regulators, it becomes toxic to the cell (41). Third, overexpression of the alginate system in wild-type cells may not be desirable unless precise environmental conditions, e.g., biofilm formation (25), are met or defense against extreme stress conditions is needed (5, 31, 49). In support of these considerations is the lack or only rare occurrence of mucoid variants among environmental isolates (3, 39). In clinical situations, such as in cystic fibrosis, under strong selective pressures during prolonged chronic infections, mucoid mutants that have lost the anti-σ factor MucA may be selected as the result of a compromise between survival in a lung infiltrated with phagocytic cells on one side (43) and some toxicity of the unchecked AlgU on the other side (41). The observations reported here also suggest that AlgZ exerts its negative actions on alginate production in an AlgR-dependent manner. This could be an additional security latch preventing uncontrolled alginate synthesis unless adequate environmental conditions are met.

The regulation of alginate production in *P. aeruginosa* is undoubtedly complicated. However, the understanding of this system continues to improve gradually, as evidenced in this study by the identification of the putative sensory component, AlgZ, proposed to interact with AlgR. Future analyses of the

regulation of AlgU activity by the products of the *mucABCD* genes and the control of AlgR activity by AlgZ should include investigations of interactions between the two systems as they bring about activation of the alginate system. For example, it will be of interest to examine how AlgR, in the context of its possible interactions with or modifications by AlgZ, cooperates with the AlgU-RNA polymerase holoenzyme to activate transcription from the subordinate promoters. While the control of alginate production offers a model system for investigating fundamental questions concerning regulation of gene expression by alternative sigma factors and signal transduction systems in bacteria, improved definition of these phenomena may also have a direct significance for our understanding of the course of infections in cystic fibrosis, since mucoidy represents a major pathogenic determinant expressed by *P. aeruginosa* in this disease (18, 43).

ACKNOWLEDGMENTS

We thank D. Krieg and S. Sonsteby for constructing pKRG1 and pSSZ1, respectively.

This work was supported by grant AI31139 from the National Institute of Allergy and Infectious Diseases and grant G844 from the Cystic Fibrosis Foundation. H. Yu was a Cystic Fibrosis Foundation postdoctoral fellow. M.J.S. was supported by grant SCHURR95I0 from the Cystic Fibrosis Foundation.

REFERENCES

1. **Aiba, H., F. Nakasai, S. Mizushima, and T. Mizuno.** 1989. Evidence for physiological importance of the phosphotransfer between the two regulatory components, EnvZ and OmpR, in osmoregulation of *Escherichia coli*. J. Biol. Chem. **264:**14090–14094.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Anastassiou, E. D., A. C. Mintzas, C. Kounavis, and G. Dimitracopoulos.** 1987. Alginate production by clinical nonmucoid *Pseudomonas aeruginosa*. J. Clin. Microbiol. **25:**656–659.
4. **Atkinson, M. R., and A. J. Ninfa.** 1993. Mutational analysis of the bacterial signal-transducing protein kinase/phosphatase nitrogen regulator II (NR_{II} or NtrB). J. Bacteriol. **175:**7016–7023.
5. **Boucher, J. C., J. M. Martinez-Salazar, M. J. Schurr, M. H. Mudd, H. Yu, and V. Deretic.** 1996. Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologs of the serine protease HtrA. J. Bacteriol. **178:**511–523.
6. **Brunskill, E. W., and K. W. Bayles.** 1996. Identification and molecular characterization of a putative regulatory locus that affects autolysis in *Staphylococcus aureus*. J. Bacteriol. **178:**611–618.
7. **Deretic, V., R. Dikshit, W. M. Konyecsni, A. M. Chakrabarty, and T. K. Misra.** 1989. The *algR* gene, which regulates mucoidy in *Pseudomonas aeruginosa*, belongs to a class of environmentally responsive genes. J. Bacteriol. **171:**1278–1283.
8. **Deretic, V., J. R. Govan, W. M. Konyecsni, and D. W. Martin.** 1990. Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: mutations in the *muc* loci affect transcription of the *algR* and *algD* genes in response to environmental stimuli. Mol. Microbiol. **4:**189–196.
9. **Deretic, V., and W. M. Konyecsni.** 1989. Control of mucoidy in *Pseudomonas aeruginosa*: transcriptional regulation of *algR* and identification of the second regulatory gene, *algQ*. J. Bacteriol. **171:**3680–3688.
10. **Deretic, V., J. H. J. Leveau, C. D. Mohr, and N. S. Hibler.** 1992. *In vitro* phosphorylation of AlgR, a regulator of mucoidy in *Pseudomonas aeruginosa*, by a histidine protein kinase and effects of small phospho-donor molecules. Mol. Microbiol. **6:**2761–2767.
11. **Deretic, V., M. J. Schurr, J. C. Boucher, and D. W. Martin.** 1994. Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. J. Bacteriol. **176:**2773–2780.
12. **Deretic, V., M. J. Schurr, and H. Yu.** 1995. *Pseudomonas aeruginosa*, mucoidy and chronic infection phenotype in cystic fibrosis. Trends Microbiol. **3:**351–356.
13. **DeVries, C. A., and D. E. Ohman.** 1994. Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternate sigma factor, and shows evidence for autoregulation. J. Bacteriol. **176:**6677–6687.
14. **Engelman, D. M., T. A. Steitz, and A. Goldman.** 1986. Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. Annu. Rev. Biophys. Chem. **15:**321–353.
15. **Erickson, J. W., and C. A. Gross.** 1989. Identification of the sigma subunit of *Escherichia coli* polymerase: a second sigma factor involved in high-temperature gene expression. Genes Dev. **3:**1462–1471.
16. **Flynn, J. L., and D. E. Ohman.** 1988. Use of a gene replacement cosmid vector for cloning alginate conversion genes from mucoid and nonmucoid *Pseudomonas aeruginosa* strains: *algS* controls expression of *algT*. J. Bacteriol. **170:**3228–3236.
17. **Fyfe, J. A. M., and J. R. W. Govan.** 1980. Alginate synthesis in mucoid *Pseudomonas aeruginosa*: a chromosomal locus involved in control. J. Gen. Microbiol. **119:**443–450.
18. **Govan, J. R. W., and V. Deretic.** 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. **60:**539–574.
19. **Igo, M. M., A. J. Ninfa, J. B. Stock, and T. J. Silhavy.** 1989. Phosphorylation and dephosphorylation of a bacterial transcriptional activator by a transmembrane receptor. Genes Dev. **3:**1725–1734.
20. **Kato, J., and A. M. Chakrabarty.** 1991. Purification of the regulatory protein AlgR1 and its binding in the far upstream region of the *algD* promoter in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **88:**1760–1764.
21. **Kato, J., L. Chu, K. Kitano, J. D. DeVault, K. Kimbara, A. M. Chakrabarty, and T. K. Misra.** 1989. Nucleotide sequence of the regulatory region controlling alginate synthesis in *Pseudomonas aeruginosa*: characterization of the *algR2* gene. Gene **84:**31–38.
22. **Kato, J., T. K. Misra, and A. M. Chakrabarty.** 1990. AlgR3, a protein resembling eukaryotic histone H1, regulates alginate synthesis in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **87:**2887–2891.
23. **Knutson, C. A., and A. Jeanes.** 1976. A new modification of the carbazole reaction: application to heteropolysaccharides. Anal. Biochem. **24:**470–481.
24. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydrophobic character of a protein. J. Mol. Biol. **157:**105–132.
25. **Lam, J., R. Chan, K. Lam, and J. W. Costerton.** 1980. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. Infect. Immun. **28:**546–556.
26. **Lipinska, B., S. Sharma, and C. Georgopoulos.** 1988. Sequence analysis and regulation of the *htrA* gene of *Escherichia coli*: a σ^{32}-independent mechanism of heat-inducible transcription. Nucleic Acids Res. **16:**10053–10067.
27. **Lonetto, M. A., K. L. Brown, K. E. Rudd, and M. J. Buttner.** 1994. Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase σ factors involved in the regulation of extracytoplasmic functions. Proc. Natl. Acad. Sci. USA **86:**7573–7577.
28. **Martin, D. W., B. W. Holloway, and V. Deretic.** 1993. Characterization of a locus determining the mucoid status of *Pseudomonas aeruginosa*: AlgU shows sequence similarities with a *Bacillus* sigma factor. J. Bacteriol. **175:**1153–1164.
29. **Martin, D. W., M. J. Schurr, M. H. Mudd, and V. Deretic.** 1993. Differentiation of *Pseudomonas aeruginosa* into the alginate-producing form: inactivation of *mucB* causes conversion to mucoidy. Mol. Microbiol. **9:**497–506.
30. **Martin, D. W., M. J. Schurr, M. H. Mudd, J. R. W. Govan, B. W. Holloway, and V. Deretic.** 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc. Natl. Acad. Sci. USA **90:**8377–8381.
31. **Martin, D. W., M. J. Schurr, H. Yu, and V. Deretic.** 1994. Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relationship to stress response. J. Bacteriol. **176:**6688–6696.
32. **May, T. B., and A. M. Chakrabarty.** 1994. *Pseudomonas aeruginosa*: genes and enzymes of alginate synthesis. Trends Microbiol. **2:**151–157.
33. **Mohr, C. D., and V. Deretic.** 1990. Gene-scrambling mutagenesis: generation and analysis of insertional mutations in the alginate regulatory region of *Pseudomonas aeruginosa*. J. Bacteriol. **172:**6252–6260.
34. **Mohr, C. D., N. S. Hibler, and V. Deretic.** 1991. AlgR, a response regulator controlling mucoidy in *Pseudomonas aeruginosa*, binds to the FUS sites of the *algD* promoter located unusually far upstream from the mRNA start site. J. Bacteriol. **173:**5136–5143.
35. **Mohr, C. D., J. H. J. Leveau, D. P. Krieg, N. S. Hibler, and V. Deretic.** 1992. AlgR-binding sites within the *algD* promoter make up a set of inverted repeats separated by a large intervening segment of DNA. J. Bacteriol. **174:**6624–6633.
36. **Mohr, C. D., D. W. Martin, W. M. Konyecsni, J. R. Govan, S. Lory, and V. Deretic.** 1990. Role of the far-upstream sites of the *algD* promoter and the *algR* and *rpoN* genes in environmental modulation of mucoidy in *Pseudomonas aeruginosa*. J. Bacteriol. **172:**6576–6580.
37. **Mohr, C. D., S. K. Sonsteby, and V. Deretic.** 1994. The *Pseudomonas aeruginosa* homologs of *hemC* and *hemD* are linked to the gene encoding the regulator of mucoidy AlgR. Mol. Gen. Genet. **242:**177–184.
38. **Parkinson, J. S., and E. C. Kofoid.** 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26:**71–112.
39. **Pier, G. B., D. DesJardins, R. Aquilhar, M. Barnard, and D. Speert.** 1986. Polysaccharide surface antigens expressed by nonmucoid isolates of *Pseudomonas aeruginosa* from cystic fibrosis patients. J. Clin. Microbiol. **24:**189–196.
40. **Schurr, M. J., H. Yu, J. C. Boucher, N. S. Hibler, and V. Deretic.** 1995. Multiple promoters and induction by heat shock of the gene encoding the alternative sigma factor AlgU (σ^{E}) which controls mucoidy in cystic fibrosis

isolates of *Pseudomonas aeruginosa*. J. Bacteriol. **177:**5670–5679.
41. **Schurr, M. J., D. W. Martin, M. H. Mudd, and V. Deretic.** 1994. Gene cluster controlling conversion to alginate-overproducing phenotype in *Pseudomonas aeruginosa*: functional analysis in a heterologous host and role in the instability of mucoidy. J. Bacteriol. **176:**3375–3382.
42. **Schurr, M. J., H. Yu, J. M. Martinez-Salazar, J. C. Boucher, and V. Deretic.** 1996. Control of AlgU, a member of the σ^E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. J. Bacteriol. **178:**4997–5004.
43. **Speert, D. P.** 1994. *Pseudomonas aeruginosa* infections in patients with cystic fibrosis, p. 183–236. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa* infections and treatment. Marcel Dekker, Inc., New York, N.Y.
44. **Stock, J. B., M. G. Surette, M. Levit, and P. Park.** 1995. Two-component signal transduction systems: structure-function relationships and mechanisms of catalysis, p. 25–52. *In* J. A. Hoch and T. J. Sihavy (ed.), Two-component signal transduction. ASM Press, Washington, D.C.
45. **Venturi, V., C. Ottevanger, J. Leong, and P. Weisbeck.** 1993. Identification and characterization of a siderophore regulatory gene (*pfrA*) of *Pseudomonas putida* WCS358: homology to the alginate regulatory gene *algQ* of *Pseudomonas aeruginosa*. Mol. Microbiol. **10:**63–73.
46. **Xie, Z.-D., C. D. Hershberger, S. Shankar, R. W. Ye, and A. M. Chakrabarty.** 1996. Sigma factor–anti-sigma factor interaction in alginate synthesis: inhibition of AlgT by MucA. J. Bacteriol. **178:**4990–4996.
47. **Yang, Y., and M. Inouye.** 1993. Requirement of both kinase and phosphatase activities of an *Escherichia coli* receptor (Taz1) for ligand-dependent signal transduction. J. Mol. Biol. **231:**335–342.
48. **Yu, H., M. J. Schurr, J. C. Boucher, J. M. Martinez-Salazar, D. W. Martin, and V. Deretic.** 1996. Molecular mechanism of conversion to mucoidy in *Pseudomonas aeruginosa*, p. 384–397. *In* T. Nakazawa, K. Furukawa, D. Haas, and S. Silver (ed.), Molecular biology of pseudomonads. ASM Press, Washington, D.C.
49. **Yu, H., M. J. Schurr, and V. Deretic.** 1995. Functional equivalence of *Escherichia coli* σ^E and *Pseudomonas aeruginosa* AlgU: *E. coli rpoE* restores mucoidy and reduces sensitivity to reactive oxygen intermediates in *algU* mutants of *P. aeruginosa*. J. Bacteriol. **177:**3259–3268.