JOURNAL OF BACTERIOLOGY, Feb. 1998, p. 956–968 Vol. 180, No. 4
0021-9193/98/$04.00+0

Phosphorylation-Independent Activity of the Response Regulators AlgB and AlgR in Promoting Alginate Biosynthesis in Mucoid *Pseudomonas aeruginosa*

SHENG MA,[1] UMA SELVARAJ,[2] DENNIS E. OHMAN,[1] RYAN QUARLESS,[2] DANIEL J. HASSETT,[3] AND DANIEL J. WOZNIAK[2]*

Department of Microbiology and Immunology, University of Tennessee and Veterans Administration Medical Center, Memphis, Tennessee 38163[1]; Department of Microbiology and Immunology, Bowman Gray School of Medicine at Wake Forest University, Winston-Salem, North Carolina 27157-1064[2]; and Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0524[3]

Received 3 September 1997/Accepted 16 December 1997

Overproduction of the capsular polysaccharide alginate appears to confer a selective advantage for *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients. The regulators AlgB and AlgR, which are both required as positive activators in alginate overproduction, have homology with the regulator class of two-component environmental responsive proteins which coordinate gene expression through signal transduction mechanisms. Signal transduction in this class of proteins generally occurs via autophosphorylation of the sensor kinase protein and phosphotransfer from the sensor to a conserved aspartate residue, which is present in the amino terminus of the response regulator. Recently, *kinB* was identified downstream of *algB* and was shown to encode the cognate histidine protein kinase that efficiently phosphorylates AlgB. However, we show here that a null mutation in *kinB* in a mucoid cystic fibrosis isolate, *P. aeruginosa* FRD1, did not block alginate production. The role of the conserved aspartate residue in the phosphorylation of AlgB was examined. The predicted phosphorylation site of AlgB (D59) was mutated to asparagine (N), and a derivative of an AlgB lacking the entire amino-terminal phosphorylation domain (AlgBΔ1-145) was constructed. A hexahistidine tag was included at the amino terminus of the wild-type (H-AlgB), H-AlgBΔ1-145, and mutant (H-AlgB.59N) AlgB proteins. These derivatives were purified by Ni^{2+} affinity chromatography and examined for in vitro phosphorylation by the purified sensor kinase protein, KinB. The results indicated that while KinB efficiently phosphorylated H-AlgB, no phosphorylation of H-AlgBΔ1-145 or H-AlgB.D59N was apparent. An allelic exchange system was developed to transfer mutant *algB* alleles onto the chromosome of a *P. aeruginosa algB* mutant to examine the effect on alginate production. Despite the defect in AlgB phosphorylation, *P. aeruginosa* strains expressing AlgB.D59N or H-AlgBΔ1-145 remained mucoid. The roles of the conserved aspartate residues in the phosphorylation of AlgR were also examined. As seen with AlgB, mutations in the predicted phosphorylation site of AlgR (AlgR.D54N and AlgR.D85N) did not affect alginate production. These results indicate that in vivo phosphorylation of AlgB and AlgR are not required for their roles in alginate production. Thus, the mechanism by which these response regulators activate alginate genes in mucoid *P. aeruginosa* appears not to be mediated by conventional phosphorylation-dependent signal transduction.

Cystic fibrosis (CF) is a common, serious, and often fatal genetic disease characterized by oversecretion of pulmonary mucus, bacterial infections, respiratory congestion, and, in many cases, death due to respiratory failure. Although the lungs of CF patients are colonized by several microorganisms, infections by *Pseudomonas aeruginosa* are the most common, are usually chronic, and are the most serious in terms of clinical prognosis (15, 23). *P. aeruginosa* isolates from such chronic infections often have a mucoid colony appearance. This phenotype is due to the overproduction and secretion of a capsular polysaccharide called alginate which plays an important role in chronic *P. aeruginosa* infections in CF patients (for a review, see reference 23).

Alginate production is controlled by a complex regulatory hierarchy involving several genes (65). A key element in alginate gene regulation is the alternative sigma factor σ^{22} (alternatively known as AlgT and AlgU), which is a member of the RpoE family of extracytoplasmic function sigma factors (13, 30). The activity of σ^{22} appears to be modulated by the *mucABCD* gene products, which are encoded by the *algT* gene operon at 68 min on the *P. aeruginosa* chromosome (23, 34, 41). Many mucoid *P. aeruginosa* isolates derived from CF patients harbor mutations in *mucA* (32), and inactivation of *mucA* or *mucB* (also referred to as *algN*) in wild-type nonmucoid *P. aeruginosa* strains causes induction of alginate synthesis (20, 31, 32). A membrane complex formed by MucA-MucB may be involved in regulating the stability of σ^{22} in the cell (34). Biochemical data show that MucA has an affinity for σ^{22} (50, 66). Active σ^{22} induces the expression of at least four genes or operons which are required for alginate synthesis. These include the *algT* operon (13, 33), the *algD* operon encoding most of the genes required for alginate synthesis (8, 11, 65), *algR* (33, 65), and the *algB* operon (29, 64, 65).

The *algB* and *algR* genes encode proteins that have homology to response regulators of the two-component superfamily (44). Both AlgR and AlgB control alginate levels by activating transcription of *algD*, the first gene of the alginate biosynthetic operon located at 34 min on the *P. aeruginosa* chromosome (8, 11, 65). AlgR activates *algD* expression directly by binding to

* Corresponding author. Phone: (336) 716-2016. Fax: (336) 716-9925. E-mail: dwozniak@bgsm.edu.

three sites, two of which are located unusually far upstream of the *algD* transcription start site (25, 37).

The mechanism by which AlgB stimulates *algD* transcription and alginate production is unclear. AlgB shows homology to response regulators of the NtrC subfamily (63). Although AlgR contains a conserved amino-terminal phosphorylation domain typical of response regulators, its output domain does not appear to fall into a known subfamily (10).

Response regulators generally have a cognate sensor kinase protein that responds to environmental stimuli and undergoes autophosphorylation at a histidine residue. The phosphate is then transferred to an aspartate residue in the amino-terminal domain of the response regulator. This phosphorylation usually activates the response regulator leading to an adaptive response. This kind of phosphorelay is a general mechanism for the activation of response regulators of two-component regulatory systems (for a review, see reference 43). Recently, a gene downstream of *algB*, designated *kinB*, was identified to encode the cognate sensor kinase for AlgB (29). The KinB protein was localized to the membrane, and a purified carboxyl terminus of KinB was able to undergo autophosphorylation and to phosphorylate AlgB (29). Upstream of *algR* is *fimS*, a gene involved in type 4 pilus-mediated twitching motility that encodes an atypical sensor protein (62); it has also been termed *algZ* (68).

Despite the evidence that KinB-AlgB and FimS-AlgR are cognate sensor response regulator pairs, it has not been established that phosphorylation of AlgB or AlgR is required for alginate production in vivo. This is a clinically important question, since it has been proposed that inhibitors of two-component signal transduction systems might have therapeutic value for CF patients colonized with *P. aeruginosa* (46). In the present study, *P. aeruginosa* strains with mutations in *kinB*, *algB*, and *algR* were constructed to test the role of phosphorylation and signal transduction in alginate synthesis. These studies showed that the response regulators AlgB and AlgR did not require phosphorylation in order to promote alginate production in mucoid *P. aeruginosa*. This suggests that an alternative and unusual mechanism may be used by these response regulators to activate gene expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *P. aeruginosa* strains used in this study are listed in Table 1. *Escherichia coli* JM109 (Promega) and XL1-Blue (Stratagene) were used for most plasmid manipulations. Bacteria were cultured in L broth (10.0 g of tryptone, 5.0 g of yeast extract, 5.0 g of sodium chloride per liter [pH 7.5]) or on L agar (Difco) plates. The media used for selection of *P. aeruginosa* and counterselection of *E. coli* following triparental mating were either a 1:1 mix of L agar and Pseudomonas Isolation Agar (Difco) or L agar lacking sodium chloride plus irgasan (Irgasan DP300; Ciba Geigy) at a final concentration of 25 μg/ml. Sucrose plates (for *sacB*-mediated counterselection) contained sucrose at a concentration of 5% (wt/vol) in L agar lacking sodium chloride, and the cultures were incubated at 30°C for 24 h. Selective antibiotics were used at the following concentrations for *P. aeruginosa*: carbenicillin, 300 μg/ml; gentamicin, 100 μg/ml; and tetracycline, 100 μg/ml. For *E. coli*, the concentrations were as follows: ampicillin, 100 μg/ml; gentamicin, 15 μg/ml; and tetracycline, 15 μg/ml. Mercuric chloride was used at 18 μg/ml for both *P. aeruginosa* and *E. coli*. Chemicals were purchased from Sigma unless stated otherwise.

Plasmids and DNA manipulations. The plasmids and oligonucleotides used in the study are listed in Table 1. Restriction enzymes were purchased from Boehringer Mannheim, Promega, or New England Biolabs. Protocols for routine cloning were described elsewhere (1, 63). Triparental matings as described previously (21, 65) were used to mobilize plasmids into *P. aeruginosa*. DNA sequences from plasmid DNA were determined by the dideoxy chain-termination method as described previously (63) with minor modifications. PCRs were performed as described elsewhere (3). Oligonucleotide-directed mutagenesis was performed with the Altered Sites Mutagenesis system (Promega), pALTER-1, and mutagenic oligonucleotides (Table 1) as described by the manufacturer. Plasmid pDJW148 was mutagenized with oligonucleotides *algB*45 and *algB*48 to generate the *algB*45 and *algB*48 alleles, respectively, and the resulting plasmids were designated pUS4 (*algB*45) and pUS5 (*algB*48). To place *Bgl*II sites flanking *algB*, pDJW17 was mutagenized with oligonucleotides *algB*50 and *algB*51 to generate pUS61. To place *Bgl*II sites flanking *algR*, pDJW106 was mutagenized with oligonucleotides *algR*5 and *algR*6 to generate pDJW385.

Determination of the sites of Tn*501* insertion in *algB* and *kinB*. The exact position of the *algB*::Tn*501-2* in FRD444 was determined by sequence analysis and shown to be inserted following bp 1034 of the *algB* open reading frame. By restriction analysis and Southern hybridization, pJG1::Tn*501-49* (22) was shown to have a Tn*501* insertion ~300 bp into the *kinB* open reading frame. *P. aeruginosa* genomic DNAs from FRD1 and FRD1049 (with Tn*501-49* in the chromosome) were isolated as described previously (21). Tn*501* has *Eco*RI sites at both its termini, which were used for mapping. The DNAs were digested with *Eco*RI and *Cla*I, subjected to electrophoresis on 0.7% agarose (SeaKem; FMC), and transferred to a nylon membrane (Boehringer Mannheim) by the capillary transfer procedure described elsewhere (1). A 280-bp digoxigenin-labeled probe, matching *kinB* sequences 5′ to the *Eco*RI site, was synthesized from pDJW130 by PCR with oligonucleotide primers P7 and P10 (Table 1) following a prior protocol (27, 29). Hybridization and detection were performed with the Genius system (Boehringer Mannheim), which revealed 1.2- and 0.9-kb bands in FRD1 and FRD1049, respectively.

Analysis of *cat* transcriptional fusions. Extracts of *P. aeruginosa* containing pSM53 (*kinB-cat*) were obtained as previously described (63). Extracts were assayed for protein concentrations by the Bradford method (7) and were assayed for chloramphenicol acetyltransferase (CAT) levels by an enzyme-linked immunosorbent assay as indicated by the manufacturer (5 Prime→3 Prime, Inc., Boulder, Colo.). CAT levels in dilutions of the cell extracts were determined by extrapolation from a standard curve and were normalized for protein content. The values were expressed as picograms of CAT per microgram of extract protein and are averages from three independent experiments.

Allelic exchange techniques. A *kinB*::Tn*501-49* mutant of FRD1 was generated with PAO1(pJG1::Tn*501-49*) and phage F116L to transfer plasmid DNA fragments by a transduction-mediated gene replacement technique as previously described (42). Mutants with altered *algB* alleles were generated by gene replacement with suicide plasmids containing *sacB* for counterselection. A schematic representation of the allele replacement technique is illustrated in Fig. 5. In order to generate the intermediate strain FRD840 (Δ*algB*::Ω*aacC1*) used as a recipient for most gene replacements at *algB*, single-stranded DNA from JM109/pDJW17 was subjected to site-directed mutagenesis with oligonucleotides *algB*50 and *algB*51. This was performed to introduce *Bgl*II cloning sites 5′ and 3′ of the *algB* coding sequence due to the lack of convenient restriction sites. The positions of these sites were important, since all desired *algB* mutations had to be contained within the *Bgl*II restriction sites (see below). The resulting plasmid (pUS61) was cleaved with *Bgl*II, and a 1.5-kb Ω*aacC1* cassette (encoding resistance to gentamicin [Gm^r]) derived from pUCGM by treatment with *Bam*HI was used to replace *algB* to form pUS63. The Δ*algB*::Ω*aacC1* allele with flanking sequences was subcloned into pEX100T, a ColE1 carbenicillin resistance (Cb^r) vector used for allelic exchange in *P. aeruginosa* (53). pEX100T can be propagated in *E. coli* but cannot replicate in *P. aeruginosa*. This vector has an *oriT* sequence which allows for pRK2013-mediated transfer from *E. coli* to *P. aeruginosa*. In addition, pEX100T contains the *sacB* gene, allowing for counterselection when *P. aeruginosa* strains containing *sacB* are cultured in the presence of sucrose (51). The subsequent plasmid (pUS65) was transferred to *P. aeruginosa* FRD1 (Alg$^+$), and colonies were selected for Gm^r (see Fig. 5A). Since pEX100T cannot replicate in *P. aeruginosa*, the only way in which a Gm^r colony can arise is through homologous recombination between sequences on the chromosome and sequences flanking *algB* on pUS65. Most Gm^r colonies were also Cb^r and contained both wild-type and Δ*algB*::Ω*aacC1* alleles, indicating single recombination events (merodiploids). To generate the second recombination, a Gm^r merodiploid strain was cultured overnight and aliquots were plated on media containing gentamicin (selectable marker) and sucrose (counterselectable marker). These sucrose-resistant, Gm^r bacteria were screened for loss of Cb^r, and introduction of the Δ*algB*::Ω*aacC1* mutant allele was verified by PCR and Southern hybridizations of chromosomal DNA (data not shown). Techniques similar to those outlined above were used to generate FRD831 (Δ*algR*::Ω*aacC1*), an intermediate strain used for *algR* allele replacements, except that the gene replacement plasmid pDJW389 used to create the intermediate strain was derived from pEMR-ST (36) rather than pEX100T. To introduce specific *algB* alleles (e.g., mutation *algB*45 [see Fig. 5B]), the reverse procedure was utilized, relying on regions of homology flanking *algB. algB* alleles (plus flanking sequences, e.g., pUS50 [see Fig. 5B]) were subcloned into pEX100T and transferred to FRD840, and the transconjugants were plated on carbenicillin plates to select for merodiploids. These Cb^r Gm^r, sucrose-sensitive bacteria were plated on sucrose media to select for the second recombination. This provided a direct selection for allele replacement and introduction of the *algB* mutation into the chromosome. As a second screen, the sucrose-resistant bacteria were tested for sensitivity to carbenicillin and gentamicin. Thus, the final strain contained the desired single-copy allele at the *algB* locus and did not require antibiotic selection. Introduction of each mutation was verified by PCR amplification of mutant chromosomal DNA followed by DNA sequence analysis of the PCR product (data not shown).

Most pEX100T-derived plasmids were generated from pALTER-1 derivatives (Table 1). Plasmid pUS50 was derived by cleaving pUS4 with *Hin*dIII-*Bam*HI treatment with T4 polymerase and subcloning the 3.9-kb fragment into the

TABLE 1. *P. aeruginosa* strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Characteristic(s) or sequence	Source or reference
E. coli		
JM109	*endA1 recA1 gyrA96 thi hsdR17* (r_{K^-} m_{K^+}) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ZΔM15]	Promega
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{q}ZΔM15 Tn*10*]	Stratagene
BMH71-18 *mutS*	*thi supE lac-proAB mutS*::Tn*10* [F′ *proAB lacI*^{q}ZΔM15]	Promega
TB1	*ara* Δ(*lac-proAB*) *rspL* ϕ80 *lacZ*ΔM15 *hsdR*	Bethesda Research Laboratories
BL21 (λDE3)	F$^-$ *ompT* [*lon*] *hsdS*$_B$(r_{B^-} m_{B^-}; B strain); λDE3 lysogen with T7 RNA polymerase	Novagen
P. aeruginosa		
FRD1	CF isolate; Alg$^+$ (due to *mucA22* mutation)	40
FRD2	*algT18* Alg$^-$	13, 40
FRD440	*algT*::Tn*501*	18
FRD444	*algB*::Tn*501*	22
FRD831	Δ*algR*::Ω*aacC1*	This study
FRD833	*algR*$^+$ derivative of FRD831	This study
FRD836	*algR*7 (encoding AlgR.D54N)	This study
FRD838	*algR*10 (encoding AlgR.D85N)	This study
FRD839	*algR*11 (encoding AlgR.D54N.D85N)	This study
FRD840	*algB*Δ::Ω*aacC1*	This study
FRD842	*algB*48 (encoding AlgB.R442E)	This study
FRD844	*algB*45 (encoding AlgB.D59N)	This study
FRD846	*algB*$^+$ derivative of FRD840	This study
FRD848	*algB*22 (encoding H-AlgB)	This study
FRD850	*algB*23 (encoding H-AlgBΔ1-145)	This study
FRD1049	*kinB*::Tn*501*	This study
Plasmids		
pALTER-1	ColE1 Tc^r Amp^s f1*ori*	Promega
pRK2013	ColE1-Tra(RK2)$^+$ Km^r	17
pUC18	ColE1 Amp^r	57
pLAFR3	IncP1 Tc^r λcos *oriT*	54
pUC4K	ColE1 Amp^r Km^r	Pharmacia
pKS$^-$	ColE1 Amp^r	Stratagene
pTrcHisA	ColE1 Amp^r *lacI*^{q} P*trc* 6x His tag vector	Invitrogen
pEX100T	ColE1 Amp^r *oriT sacB*	53
pEMR-ST	ColE1 Tc^r Amp^s f1*ori cos oriT sacB*	36
pUCGM	ColE1 Amp^r Gm^r Ω*aacC1*	52
pAB2001	ColE1 Amp^r Gm^r	4
pSM14	pJG222 + 0.8-kb *Hin*dIII fragment of *cat*; *kinB-cat* transcriptional fusion	This study
pSM50	pSM14 with *Kpn*I-*Age*I fragment deleted	This study
pSM53	pSM50 + 1.6-kb *Hin*dIII fragment containing *oriT*	This study
pSM95	ColE1 Km^r pT7 His-KinB	29
pAL1	IncP1 Tc^r pCP13 + 20-kb *Hin*dIII fragment; *algR*$^+$	65
pJG1	IncP1 Tc^r pLAFR1 + 20-kb *Eco*RI fragment; *algB*$^+$ *kinB′*	21
pJG1::Tn*501*-49	pJG1 with *kinB*::Tn*501*	22
pDJW15	pKS$^-$ + 3.9-kb *Hin*dIII-*Xho*I fragment; *algB*$^+$	64
pDJW17	pALTER-1 + 4.3-kb *Hin*dIII-*Eco*RI fragment; *algB*$^+$	This study
pDJW52	pKK223-3 + p*tac-algB*; Amp^r	63
pDJW130	pKS$^-$ + 0.9-kb *Xho*I-*Eco*RI fragment of pJG1; *′kinB*	This study
pDJW148	pALTER-1 + 3.9-kb *Hin*dIII-*Xho*I fragment; *algB*$^+$	This study
pDJW385	pDJW106 mutagenized with *algR*5 and *algR*6	This study
pDJW387	pDJW385 Δ*Bgl*II + 1.5-kb *Bam*HI of pUCGM; Δ*algR*::Ω*aacC1*	This study
pDJW389	pEMR-ST + 3.7-kb *Sal*I of pDJW387; Δ*algR*::Ω*aacC1*	This study
pDJW400	pUC18 + 1.3-kb *Bam*HI-*Eco*RI PCR fragment; *algB*22	This study
pDJW403	pTrcHisA + 1.3-kb *Bam*HI-*Eco*RI fragment of pDJW400; *algB*22	This study
pDJW406	pUC18 + 800-bp *Bam*HI-*Eco*RI PCR fragment; *algB*23	This study
pDJW408	pTrcHisA + 800-bp *Bam*HI-*Eco*RI PCR fragment; *algB*23	This study
pDJW430	pALTER-1 + 3.0-kb *Sph*I fragment of pDJW403; *algB*22	This study
pDJW433	pALTER-1 + 2.6-kb *Sph*I of pDJW408; *algB*23	This study
pDJW437	pAB2001 + 3.0-kb *Sph*I fragment of pDJW430; *algB*22	This study
pDJW438	pAB2001 + 2.6-kb *Sph*I of pDJW433; *algB*23	This study
pDJW470	pUS68 + 3.1-kb *Kpn*I-*Xho*I of pDJW437; *algB*22	This study
pDJW471	pUS68 + 2.7-kb *Kpn*I-*Xho*I of pDJW438; *algB*23	This study
pDJW525	ColE1 Amp^r Km^r *oriT sacB*	This study
pDJW527	ColE1 Amp^r Gm^r *oriT sacB*	This study
pUS4	pDJW148 mutagenized with oligonucleotide *algB*45	This study
pUS5	pDJW148 mutagenized with oligonucleotide *algB*48	This study
pUS14	pLAFR3 + *Hin*dIII-*Bam*HI fragment of pUS4; *algB*45	This study

Continued on following page

TABLE 1—*Continued*

Strain, plasmid, or oligonucleotide	Characteristic(s) or sequence	Source or reference
pUS50	pEX100T + 3.9-kb *Hin*dIII-*Xho*I fragment of pUS4; *algB*45	This study
pUS51	pEX100T + 3.9-kb *Hin*dIII-*Xho*I fragment of pUS5; *algB*48	This study
pUS55	pUC18 + 1.3-kb *Bam*HI-*Eco*RI PCR fragment; *algB*45	This study
pUS56	pTrcHisA + 1.3-kb *Bam*HI-*Eco*RI fragment of pUS55; *algB*45	This study
pUS61	pDJW17 mutagenized with oligonucleotides *algB*50 and *algB*51	This study
pUS63	pUS66 + 1.5-kb *Bam*HI fragment of pUCGM; Δ*algB*::Ω*aacC1*	This study
pUS65	pEX100T + 5.1-kb *Eco*RI-*Cla*I fragment of pUS63; Δ*algB*::Ω*aacC1*	This study
pUS66	pUS61 cleaved with *Bgl*II and the 1.8-kb *algB* fragment deleted; Δ*algB*	This study
pUS68	pEX100T + 2.9-kb *Hin*dIII-*Eco*RI fragment of pUS66; Δ*algB*	This study
pUS69	pUS68 + 2.1-kb *Kpn*I-*Xho*I fragment of pDJW17; $algB^+$	This study
pUS150	pEX100T + 2.7-kb *Sal*I fragment from pDJW106; *algR*	This study
pUS152	pDJW106 mutagenized with oligonucleotide *algR*7	This study
pUS157	pEX100T + 2.7-kb *Sal*I fragment from pUS152; *algR*7	This study
pUS164	pDJW106 mutagenized with oligonucleotide *algR*10	This study
pUS165	pDJW106 mutagenized with oligonucleotides *algR*7 and *algR*10; *algR*11	This study
pUS166	pDJW525 + 2.7-kb *Sal*I fragment from pUS164; *algR*10	This study
pUS168	pDJW525 + 2.7-kb *Sal*I fragment from pUS165; *algR*11	This study
Oligonucleotides		
*algB*45	GGCGCAGGTTGAGGAAGCA[a]	This study
*algB*48	TGCTTGCGCTTCTCGTACAGGGTCGA[a]	This study
*algB*50	TTCGCTACCCAGATCTGCCTGGCCCC[a]	This study
*algB*51	ACCGGGTCCAGATCTTCATCGG[a]	This study
*algB*52	CGGGATCCATGGAAACCACTTCC	This study
*algB*53	CGGAATTCATCGGCAGCGGC	This study
*algB*54	CGGGATCCTGGAATCGCACAGGCC	This study
*algR*5	CGGCACTAGATCTGTCTACG[a]	This study
*algR*6	GCATCAGATCTGACGGC[a]	This study
*algR*7	GTCCTGCTGAATATCCGCA[a]	This study
*algR*10	ACGGCCCATAACGAATTCG[a]	This study
*algR*11	GAAGCGCTGACGCTGAT	This study
*algR*12	TCGCTGCGCACCGGCTTGA	This study
*kinB*P7	CTCGACAGCATCGACGACGG	This study
*kinB*P10	CGCTATGCCCGCCGGGATGG	This study

[a] Mutagenic oligonucleotide (underlined residue[s] represents altered nucleotide).

unique *Sma*I site of pEX100T. This plasmid was mobilized into *P. aeruginosa* FRD1, and allele replacements were performed as above, generating strain FRD844. Similar approaches were used to produce strain FRD842 from pUS51 (*algB*48). Some allele replacements were performed with derivatives of pUS68. Plasmid pUS69 (wild-type *algB*) was constructed simply by subcloning the 2.1-kb *Kpn*I-*Xho*I fragment from DJW17 into similarly digested pUS68. pUS69 was used to generate *P. aeruginosa* FRD846. *P. aeruginosa* FRD848 (*algB*22 [H-AlgB]) and FRD850 (*algB*23 [H-AlgBΔ1-145]) were constructed by similar allelic exchange techniques with plasmids pDJW470 and pDJW471, respectively. For gene replacements involving *algR*, the intermediate strain FRD831 (Δ*algR*::Ω*aacC1*) was utilized with pEX100T- or pDJW525-derived plasmids harboring wild-type or mutant *algR* alleles. These plasmids, which included pUS150 (wild-type *algR*), pUS157 (*algR*7), pUS166 (*algR*10), and pUS168 (*algR*11), were used in allele replacements of FRD831 to generate *P. aeruginosa* FRD833, FRD836, FRD838, and FRD839, respectively.

Purification of AlgB and H-AlgB proteins. AlgB was overproduced in *E. coli* XL1-Blue(pDJW52) and purified by streptomycin sulfate precipitation, precipitation with 30% ammonium sulfate, and DEAE anion-exchange chromatography essentially as previously described (29). Approximately 40 μg of this AlgB preparation (>90% pure) was concentrated with an Applied Biosystems Pro Spin Sample Preparation Cartridge, and the polyvinylidene difluoride membrane containing AlgB was subjected to direct amino-terminal sequence analysis. The sequence was found to be Glu-Thr-Thr-Ser-Glu-Lys-Gln-Gly-Arg-Ile-Leu, which is the same as that deduced from previous DNA sequence analysis of *algB* (63).

H-AlgB fusion proteins were expressed and purified from *E. coli* JM109 containing either pDJW403 (H-AlgB), pUS56 (H-AlgB.D59N), or pDJW408 (H-AlgBΔ1-145). DNA encoding H-AlgB or H-AlgB.D59N was obtained by PCR amplification of plasmids containing wild-type *algB* (pDJW148) or *algB*45 (pUS4) with primers *algB*52 and *algB*53. The 1.3-kb fragments from the PCR amplification products were cloned into pUC18 as *Bam*HI-*Eco*RI fragments resulting in pDJW400 (wild-type *algB*) or pUS55 (*algB*45), and the DNA sequences of the PCR-generated fragments were determined and shown to be identical to pDJW148 or pUS4 sequences, respectively (data not shown). The *Bam*HI-*Eco*RI fragments of pDJW400 or pUS5 were subcloned into pTrcHisA (Invitrogen) to generate pDJW403 and pUS56, respectively. Similar approaches were used to clone DNA expressing H-AlgBΔ1-145, except that oligonucleotide *algB*54 was substituted for *algB*52 in the PCR of pDJW148. The 0.8-kb *Bam*HI-*Eco*RI PCR fragment was cloned into pUC18 (pDJW406), and the sequence was determined to be identical to that of pDJW148. The *Bam*HI-*Eco*RI fragment of pDJW406 was subcloned into pTrcHisA to generate pDJW408. pTrcHisA is an expression vector which contains the P*tac* promoter with a *lac* operator sequence, the $lacI^q$ gene, and a multicloning site. When the *Bam*HI-*Eco*RI fragments described above were cloned into pTrcHisA, the resulting plasmids expressed fusion proteins consisting of an amino-terminal (~3-kDa) peptide sequence derived from bacteriophage T7 coat protein and an additional stretch of six histidine residues. The hexahistidine tag allowed for purification of the H-AlgB proteins by nickel agarose chromatography (Qiagen). For purification of the H-AlgB proteins, 500-ml cultures of JM109 harboring pDJW403, pUS56, or pDJW408 were cultured in L broth plus ampicillin to an A_{600} of 0.3. Isopropyl-β-D-thiogalactopyranoside was added to a concentration of 1 mM, and the cells were cultured for an additional 3 h, harvested by centrifugation, and suspended in 5 ml of fractionation buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM $MgCl_2$). Cell extracts were prepared by subjecting the mixture to a French press (15,000 lb/in^2) followed by centrifugation. H-AlgB proteins were purified under native conditions from the supernatant fraction by nickel agarose chromatography as outlined by the manufacturer of the Ni-nitrilotriacetic acid agarose resin (Qiagen). Approximately 1 mg of pure H-AlgB was obtained per 500 ml of culture.

Immunoblot analysis. Polyclonal antisera against AlgB were elicited in New Zealand White rabbits (Immunodynamics, Inc.) with purified AlgB protein (0.75 mg). Anti-AlgB antibodies were used in immunoblots at a dilution of 1:20,000 with chemiluminescent reagents by procedures outlined by the manufacturer (Amersham), and film was exposed for 30 s prior to development.

In vitro phosphorylation assays. The conditions used in the autophosphorylation of KinB and phosphotransfer from KinB to AlgB have been described previously (29). Briefly, the cytoplasmic carboxy terminus of KinB (1.3 μM) was incubated with 33.3 μM [γ-^{32}P]ATP for 60 min at room temperature in a buffer containing 50 mM KCl and 5 mM $MgCl_2$. H-AlgB protein (3.0 μM) was added to the mixture, which was further incubated for 60 s. The reaction was terminated

by adding sodium dodecyl sulfate (SDS) sample buffer (60 mM Tris hydrochloride [pH 6.8], 2% SDS, 10% glycerol, 0.1 mg/ml bromphenol blue, 5% 2-mercaptoethanol), unincorporated label was removed, and the products were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. For competition assays, kinase reactions were performed as described above, except that H-AlgB.D59N or H-AlgBΔ1-145 was included in the mixture at concentrations of 1.3, 5.2, or 13.0 μM.

Alginate assays. Alginates were collected from cultures grown in L broth with rapid aeration at 37°C for 22 h, and levels were determined as previously outlined (26), with modifications (19). Briefly, samples (5 ml) of cultures were mixed with 5 ml of saline, and the cells were removed by centrifugation (12,000 × *g* for 30 min). The culture supernatant was mixed with 5 ml of 2% cetyl pyridinium chloride, and the precipitated alginate was collected by centrifugation (12,000 × *g* for 10 min at room temperature). The pellet was dissolved in 10 ml of 1 M NaCl, precipitated again with 10 ml of cold (−20°C) isopropanol, and dissolved in 10 ml of saline. The concentration of alginate in solution was determined by the carbazole method described by Knutson and Jeanes (26), in which a solution of alginate (30 μl) was mixed with 1.0 ml of borate-sulfuric acid reagent (10 mM H_3BO_3 in concentrated H_2SO_4) and 30 μl of carbazole reagent (0.1% in ethanol). The mixture was then incubated in a 55°C bath for 30 min, and absorbance at 530 nm was determined spectrophotometrically. The alginate concentration was determined by extrapolation from a standard curve with various concentrations (0 to 50 μg/ml) of alginate (high viscosity from *Macrocystis pyrifera*).

RESULTS

The *algB* and *kinB* genes form an operon. AlgB is a two-component regulator that is required for expression of the alginate biosynthetic operon, and the level of *algB* expression is elevated in mucoid strains (64). The *kinB* gene, downstream and adjacent to *algB*, encodes a cognate histidine kinase that efficiently phosphorylates AlgB (29). To determine if *kinB* was part of the alginate regulon under σ^{22} (*algT*/*algU*) control, we examined the expression of *kinB* in Alg^+ and Alg^- *P. aeruginosa*. A *kinB-cat* transcriptional fusion in a suicide vector was constructed and integrated into the chromosomes of *P. aeruginosa* strains by single-crossover homologous recombination (Fig. 1A). Alg^+ *P. aeruginosa* FRD1 carrying *kinB-cat* (pSM53) contained CAT levels that were approximately threefold higher than that seen in the Alg^- *algT*18 mutant strain, FRD2 (Table 2). These results were similar to the expression levels of a plasmid-borne *algB-cat* fusion in these Alg^+ and Alg^- strains (64). This was not unexpected, since sequence analysis showed that the predicted translational start (ATG) for *kinB* overlaps the stop codon for *algB*, suggesting that they form an operon (29). This was further tested by examining whether the *algB* transposon insertion (*algB*::Tn*501*-2) in FRD444 was polar on the downstream *kinB* gene. The position of Tn*501*-2 in *algB* was determined by sequence analysis and was shown to be inserted following bp 1034 of the *algB* open reading frame. Analysis of *kinB-cat* expression in Alg^- FRD444::pSM53 (where *kinB-cat* was positioned downstream of the polar Tn*501*-2 insertion [Fig. 1B]) revealed dramatically reduced *kinB* levels (Table 2). This further suggested that *algB* and *kinB* formed an operon. Furthermore, providing FRD444::pSM53 with *algB* in *trans* on pJG1 did not restore *kinB-cat* expression, indicating that *kinB* did not have an AlgB-dependent promoter. Interestingly, providing Alg^- FRD444::pSM53 with *algB* in *trans* did restore the Alg^+ phenotype, even though one would predict this strain to be *kinB* defective.

A *kinB* null mutation did not affect alginate production. The genetic data above suggested that KinB was not essential for alginate production, even though *kinB* was in an operon with *algB* and encoded its cognate kinase, which has been shown to efficiently phosphorylate AlgB in vitro (29). To directly test the role of KinB in alginate production, a *kinB*::Tn*501* mutant of Alg^+ FRD1 was constructed by gene replacement. In a prior study, plasmid pJG1 was subjected to Tn*501* transposon mutagenesis in an attempt to localize the *algB* gene (22). One of these plasmids, pJG1::Tn*501*-49, was found to carry a Tn*501* insertion within the first 300 bp of the *kinB* open reading frame and was used to generate the *kinB*::Tn*501* null mutant, FRD1049 (Fig. 1C). Interestingly, the colony morphology of FRD1049 on L agar (following incubation for 18 h at 37°C) was mucoid, and this strain synthesized alginate at levels comparable to those of the parental strain FRD1 (Fig. 2). Other mutants generated by insertional disruption of *kinB* in the FRD1 background also remained Alg^+ (data not shown). As

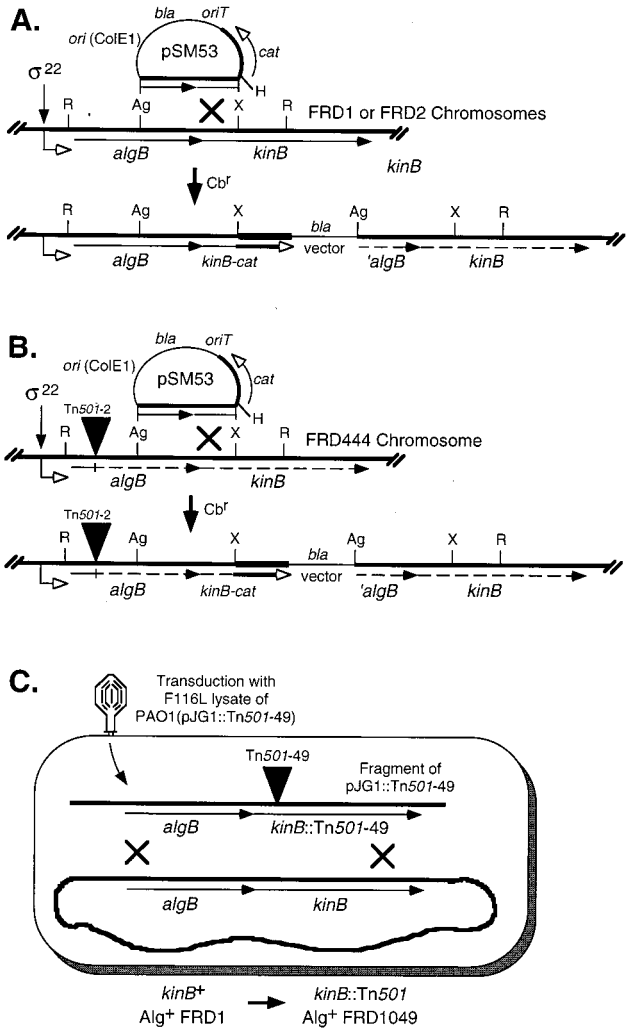

FIG. 1. Diagram of genetic constructions used to modify the chromosomal *kinB* gene in *P. aeruginosa* FRD. (A) Construction of *kinB-cat* transcriptional fusion in Alg^+ strain FRD1 and Alg^- strain FRD2. A promoterless *cat* gene cassette (0.8-kb *Hin*dIII fragment) was cloned to form a *kinB-cat* transcriptional fusion in pSM53. This plasmid has a ColE1 origin, which cannot replicate autonomously in *P. aeruginosa*, and was integrated into the chromosomes of FRD1 and FRD2 (*algT*18) by homologous recombination via selection for carbenicillin resistance encoded by *bla*. Dashed arrows indicate genes that are not transcribed due to the polar upstream insertion of the vector. (B) Construction of *kinB-cat* transcriptional fusion in Alg^- strain FRD444 (*algB*::Tn*501*-2). The Tn*501* insertion in *algB* (closed triangle) is polar on downstream genes, as indicated by dashed arrows. (C) Construction of the *kinB* mutant FRD1049. A gene replacement technique (42) was used to transfer an *kinB*::Tn*501* allele into the chromosome of *P. aeruginosa* FRD1. Briefly, a lysate of phage F116L was generated on *P. aeruginosa* PAO1(pJG1::Tn*501*-49) and used to transduce FRD1. Mercury resistance encoded by Tn*501* was used to select for double-crossover events of *kinB*::Tn*501* with the chromosome, and the strain was scored for loss of plasmid-borne tetracycline resistance. Abbreviations: Ag, *Age*I; R, *Eco*RI; H, *Hin*dIII; X, *Xho*I; *bla*, gene encoding carbenicillin resistance; *cat*, gene encoding CAT.

TABLE 2. Analysis of *kinB-cat* expression in *P. aeruginosa* strains

Strain	Genotype	Alginate phenotype	Amt of CAT (pg/μg of protein) from *kinB-cat*[a]
FRD1	Wild type	Alg^+	<10
FRD1::pSM53	*kinB-cat*	Alg^+	438 ± 31
FRD2::pSM53	*algT*18 *kinB-cat*	Alg^-	146 ± 21
FRD444::pSM53	*algB*::Tn*501 kinB-cat*	Alg^-	<10
FRD444::pSM53 (pJG1)	*algB*::Tn*501 kinB-cat* (*algB*$^+$)	Alg^+	<10

[a] *P. aeruginosa* FRD strains containing a chromosomally integrated pSM53 (*kinB-cat*) were cultured to the same density in logarithmic phase and were harvested. As a measure of relative *kinB* transcription, cell extracts of these strains were assayed for CAT levels relative to the protein concentrations.

controls, strains FRD840 (Δ*algB*::Ω*aacC1*, see below) and FRD440 (*algT*::Tn*501*) were nonmucoid and produced little if any detectable alginate (Fig. 2). Thus, a null mutation in *kinB* appeared to have no obvious effect on alginate production under the conditions tested here. This suggested that phosphorylation of AlgB by KinB was not required for alginate overproduction in mucoid *P. aeruginosa.*

Purification of AlgB derivatives predicted to have phosphorylation defects. The data above did not negate the possibility that AlgB was phosphorylated by another kinase. We then tested the possibility that AlgB activity for alginate production may require phosphorylation by a process independent of KinB kinase activity. Another histidine kinase (i.e., a cross-talk mechanism) or a small-molecular-weight phosphodonor may be sufficient for this phosphorylation reaction, and this has been proposed for other response regulators (35, 59). To address this, *algB* alleles that were predicted to encode AlgB proteins defective in phosphorylation were constructed. Based on its close relatedness to the well-studied NtrC subfamily of response regulators (38, 55, 63), AlgB was predicted to contain three functional domains (Fig. 3A): (i) an amino-terminal phosphorylation domain that is conserved across families of response regulators, (ii) a central nucleotide-binding domain that is required for facilitating transcription initiation by RNA polymerase containing σ^{54}, and (iii) a carboxy-terminal helix-turn-helix motif that is presumably involved in binding DNA sequences that are often located far upstream of the target promoter.

The phosphorylation domain was targeted here for site-directed mutagenesis. In the well-characterized response regulator CheY, three essential residues in this domain, including one lysine and two aspartate residues, form an acid pocket (56, 58), and Asp-57 within this pocket is the site of phosphorylation (48). The aspartate residue represented by Asp-57 in CheY is also the primary site of phosphorylation in NtrC, VirG, and OmpR (9, 24, 47). The three highly conserved residues in the NtrC phosphorylation domain (Asp-11, Asp-54, and Lys-104) correspond to Asp-16, Asp-59, and Lys-109, respectively, in AlgB (Fig. 3A). To investigate the site of AlgB phosphorylation, the *algB*45 allele was constructed by oligonucleotide-directed mutagenesis to encode AlgB.D59N, in which the predicted phosphorylated residue Asp-59 was changed to asparagine (Fig. 3B). Also, the *algB*23 allele was constructed encoding AlgBΔ1-145, in which the entire phosphorylation domain of AlgB (residues 1 to 145) was deleted. To facilitate purification by nickel affinity chromatography, the wild-type and two mutant AlgB proteins were produced as fusion proteins with amino-terminal tags (~3 kDa) consisting of six histidine residues and a peptide sequence derived from bacteriophage T7 coat protein. The purified His-tagged AlgB (H-AlgB) proteins showed the following expected mobilities on SDS-PAGE: 52 kDa for H-AlgB, 36 kDa for H-AlgBΔ1-145, and 52 kDa for H-AlgB.D59N (Fig. 3C-left, lanes 2, 3, and 4, respectively). For comparison, a previously described (29) 39-kDa soluble derivative of KinB (C-KinB) that lacked the amino-terminal membrane hydrophobic sequence yet retained ki-

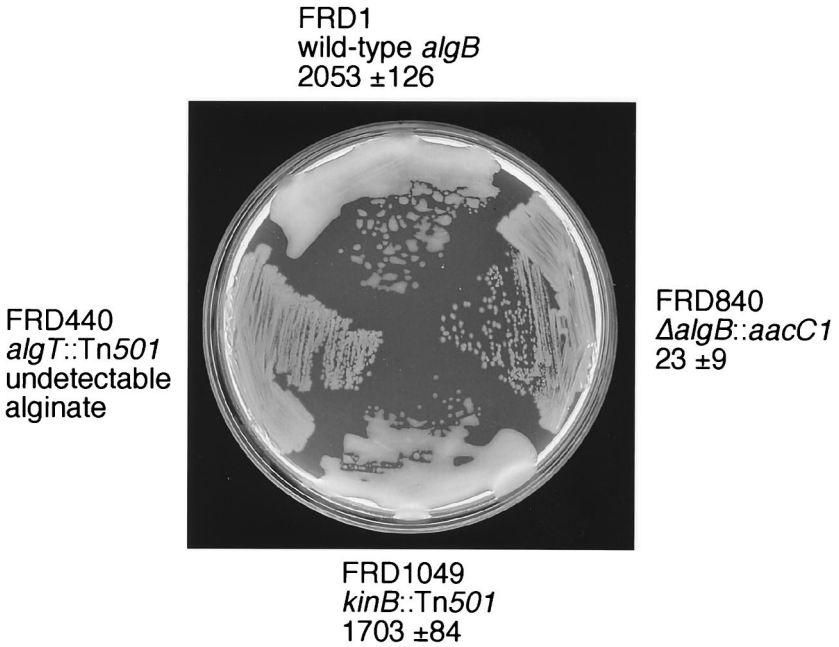

FIG. 2. Plate phenotypes of *P. aeruginosa* strains carrying wild-type or mutant *kinB* alleles. FRD1, wild type; FRD840, Δ*algB*::Ω*aacC1*; FRD1049, *kinB*::Tn*501*; and FRD440, *algT*::Tn*501* on L agar. Numbers are levels (in micrograms per milliliter) of alginate produced as determined by the carbazole assay (19, 26). Note that a *kinB* null mutation (FRD1049) did not affect alginate production.

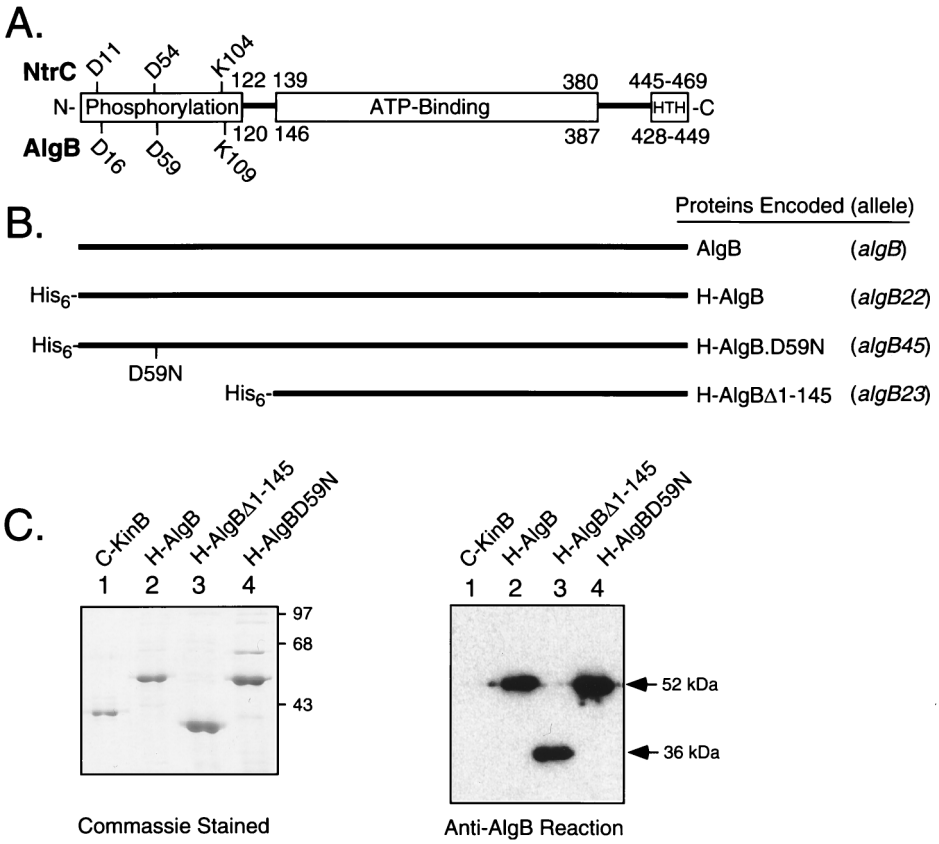

FIG. 3. (A) The modular structures of the AlgB protein and related NtrC protein are depicted. AlgB and NtrC are homologous over the entire lengths of the proteins, including a central domain with consensus ATP binding sites and the helix-turn-helix (HTH) DNA binding domain (63). Numbers above and beneath the boxes indicate positions of amino acid residues in the respective proteins. Both proteins contain a highly conserved amino-terminal domain (residues 1 to 120) which in NtrC, CheY and PhoB, OmpR, and VirG (and likely AlgB and AlgR) represents the phosphorylation domain (56). Residues corresponding to Asp13, Asp57, and Lys109 in CheY are the most highly conserved among response regulators, since these residues cluster together around the site of phosphorylation. The phosphorylation site of NtrC (Asp54 in the center of the N-terminal domain) aligns with Asp57 of AlgB and Asp54 of AlgR. (B) Depiction of AlgB proteins used in this study. The *algB* alleles which encode these proteins are indicated on the right and include *algB* (wild-type protein), *algB*22 (H-AlgB), *algB*45 (H-AlgB.D59N), and *algB*23 (H-AlgBΔ1-145). (C) Purification of H-tagged wild-type and mutant AlgB proteins. (Left) H-tagged proteins were purified and subjected to SDS-PAGE followed by Commassie blue staining. Each lane contains 1.5 μg of protein purified from *E. coli* BL21(λDE3, pSM95) (lane 1) or JM109 containing pDJW403 (lane 2), pDJW408 (lane 3), or pUS56 (lane 4). Purified C-KinB (lane 1) was included to show its relative size compared to those of AlgB protein and derivatives. Positions of protein size markers (97, 68, and 43 kDa) are indicated. (Right) Immunoblot assay of purified H-AlgB proteins. Lanes 1 to 4, preparations identical to those described for the left gel except that 200 ng of protein/lane was used. Immunoblots were performed with rabbit anti-AlgB serum, and antigen-antibody complexes were detected with enhanced chemiluminescence reagents (Amersham).

nase activity is shown (lane 1). All of the AlgB derivatives reacted with a polyclonal antiserum specific for AlgB in an immunoblot assay (Fig. 3C, right). The amino-terminal tags were not removed, because they did not appear to affect AlgB function (see below).

AlgB.D59N and AlgBΔ1-145 show defects in phosphorylation. An in vitro reaction was used to determine whether the wild-type and mutant forms of H-AlgB were capable of being phosphorylated by KinB, its cognate histidine protein kinase. KinB is a membrane protein, but the soluble and readily purified carboxyl-terminal fragment of KinB (C-KinB) has been shown to rapidly phosphorylate AlgB (29) and was used here. When C-KinB (1.3 μM) was incubated with excess [γ-^{32}P]ATP (33.3 μM), it underwent autophosphorylation (C-KinB~P; Fig. 4A, lane 4) as previously described (29). None of the other H-AlgB proteins alone showed any autophosphorylation activity (lanes 5 to 7). When purified H-AlgB protein (3.0 μM) was incubated for 60 s with C-KinB~P, most of the label transferred to H-AlgB (Fig. 4A, lane 1). Thus, the amino-terminal tag on H-AlgB did not block its phosphorylation by C-KinB~P. However, phosphorylation of H-AlgBΔ1-145 (Fig. 4A, lane 2) or H-AlgB.D59N (lane 3) was not detected. This phosphotransfer procedure was performed with a wide range of H-AlgB.D59N and H-AlgBΔ1-145 protein concentrations, yet phosphorylation of these proteins was still not observed (data not shown).

A competition assay was also performed. Compared to the phosphorylation of H-AlgB in the presence of C-KinB~P (Fig. 4B, lane 4), the addition of H-AlgB.D59N (10-fold molar excess) nearly eliminated H-AlgB phosphorylation (Fig. 4B, lane 3). However, this effect was not observed when H-AlgBΔ1-145 was used as a competitor (Fig. 4B, lanes 5 to 7). The ability of H-AlgB.D59N to inhibit the phosphorylation of H-AlgB suggests that this mutant protein still retained the ability to interact with C-KinB~P even though it could not be phosphorylated. These data provide experimental evidence that Asp-59 represents the site of AlgB phosphorylation and fulfills the prediction based on sequence homology to NtrC. Thus, substitution of this residue or deletion of the phosphorylation domain was predicted to block phosphorylation of AlgB in vivo.

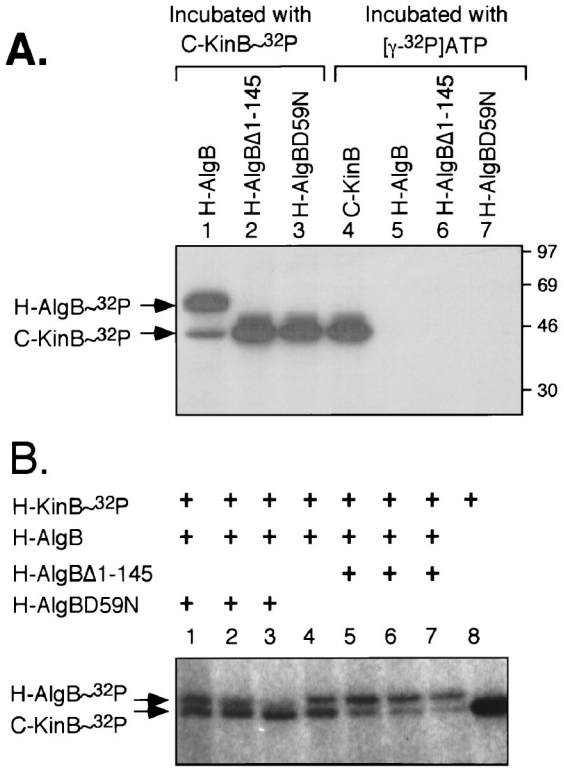

FIG. 4. Assays of in vitro phosphorylation of purified H-AlgB and derivatives by C-KinB~^{32}P. (A) C-KinB (1.3 μM) showed autophosphorylation following incubation with 33.3 μM [γ-^{32}P]ATP for 60 min (lane 4) and was then incubated for 60 s with 3.0 μM purified H-AlgB (lane 1), H-AlgBΔ1-145 (lane 2), or H-AlgB.D59N (lane 3), followed by termination of the reaction with SDS sample buffer. Unincorporated label was removed, and the samples were analyzed by SDS–10% PAGE, followed by autoradiography. As controls, H-AlgB (lane 5), H-AlgBΔ1-145 (lane 6), and H-AlgB.D59N (lane 7) were incubated with [γ-^{32}P]ATP under identical conditions for 60 s. The positions of the phosphorylated forms of C-KinB (C-KinB~P) and H-AlgB (His-AlgB~P) are indicated on the side. Note that C-KinB can phosphorylate H-AlgB but not H-AlgB.D59N or H-AlgBΔ1-145. (B) Demonstration that H-AlgB.D59N can compete with H-AlgB for interaction with C-KinB~^{32}P. (Top) schematic diagram of H-AlgB proteins used in the competition assay (+, addition of that protein). C-KinB was autophosphorylated as described above, and aliquots (1.3 μM) were removed and added to SDS-PAGE sample buffer (lane 8) or to a sample containing H-AlgB (3.0 μM) either alone (lane 4) or with increasing amounts of H-AlgB.D59N (lane 1, 1.3 μM; lane 2, 5.2 μM; lane 3, 13.0 μM H-AlgB.D59N addition) or H-AlgBΔ1-145 (lane 5, 1.3 μM; lane 6, 5.2 μM; lane 7, 13.0 μM H-AlgBΔ1-145 addition). Kinase reactions were allowed to proceed and analyzed as described for A.

AlgB derivatives blocked in phosphorylation still promoted alginate production in mucoid *P. aeruginosa*. To test the potential role of AlgB phosphorylation in alginate gene activation, the *algB*45 allele (encoding AlgB.D59N but lacking a His tag) was cloned onto pLAFR3, a low-copy-number, broad-host-range plasmid, to form pUS14, which was transferred to Alg$^-$ FRD444, an *algB*::Tn*501* mutant. Although AlgB.D59N was shown above to be defective in phosphorylation, the transconjugates obtained were complemented and displayed the Alg$^+$ phenotype (data not shown). This suggested that AlgB may function to promote alginate overproduction without phosphorylation. However, we could discount the possibility that this result was due to the multiple copies of plasmid-borne *algB* alleles in the cell.

To avoid the possibility of gene dosage effects, gene replacement was used to place mutant alleles onto the *P. aeruginosa* FRD1 chromosome so that they could be tested in single copy under native transcriptional and translational controls (see Materials and Methods). To test the role of AlgB phosphorylation, we constructed strain FRD844 through a two-step process in which the wild-type *algB* allele was exchanged for a Ω*aacC1* cassette encoding Gm^r and then by the *algB*45 allele, which encoded AlgB.D59N (Fig. 5). As a control, FRD846 was constructed by the same two-step process to restore the wild-type *algB* allele. FRD842 was constructed with the *algB*48 allele, which encoded AlgB.R442E. In addition, FRD848 (*algB*22 encoding H-AlgB) and FRD850 (*algB*23 encoding H-AlgBΔ1-145), in which the *algB* alleles were integrated into the chromosome, expressed under the vector's promoter, and produced His-tagged proteins, were constructed.

The presence of a single copy of *algB* in the chromosomes of each of these strains was confirmed by Southern hybridization (data not shown). An immunoblot analysis demonstrated AlgB-reactive bands of anticipated sizes in all of these strains, and proteins appeared to be at a level similar to that in wild-type FRD1 when expressed under the native *algB* promoter (Fig. 6). Also, the mutant alleles introduced encoded proteins that appeared to have approximately the same susceptibilities to endogenous proteolytic degradation in the cell extracts as wild-type AlgB, which suggests that the substitutions had little effect on the overall structures of the AlgB proteins (Fig. 6).

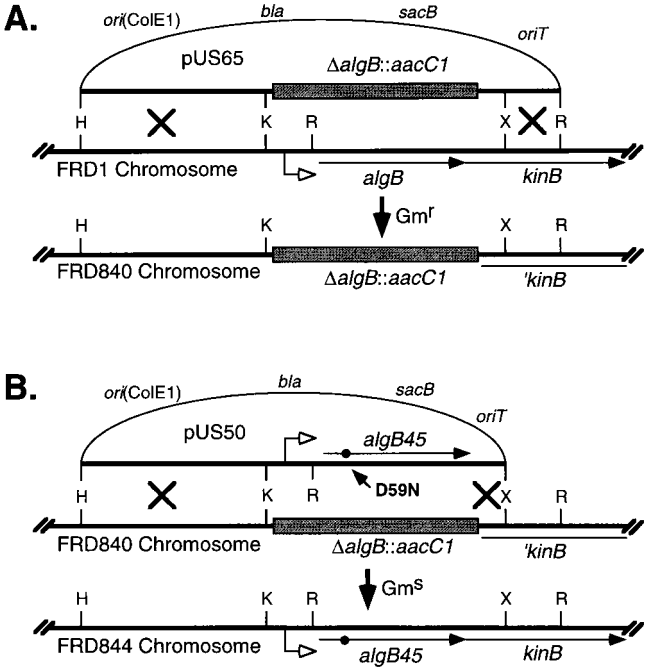

FIG. 5. Depiction of the two-step procedure used to transfer *algB* alleles to the *P. aeruginosa* chromosome by allelic exchange. (A) Generation of Δ*algB*::Ω*aacC1* intermediate strain FRD840. Plasmid pUS65 (pEX100T plus Δ*algB*::Ω*aacC1*), which cannot replicate in *P. aeruginosa*, was mobilized into strain FRD1 and single-crossover recombinants were isolated by selection for Gm^r. Most Gm^r bacteria were also Cb^r, indicating integration of the entire plasmid (single-crossover events). Double recombinants were isolated by plating Gm^r bacteria on agar containing 5% sucrose. The desired (double) recombinants were Gm^r Cb^s (marker on pUS65). (B) Allele replacements. To perform allele replacements, pEX100T containing a specific *algB* allele (*algB*45 in this example) was mobilized into the intermediate strain FRD840, and single recombinants were selected by resistance to carbenicillin. The desired allele replacements (Gm^s Cb^s) were then obtained by counterselection on sucrose-containing medium. Abbreviations: *ori*, ColE1 origin; *sacB*, gene encoding levansucrase; *oriT*, transfer origin; *bla*, resistance to carbenicillin; *aacC1*, resistance to gentamicin; s, antibiotic sensitivity; Cb, carbenicillin; Gm, gentamicin; H, *Hin*dIII; K, *Kpn*I; R, *Eco*RI; X, *Xho*I.

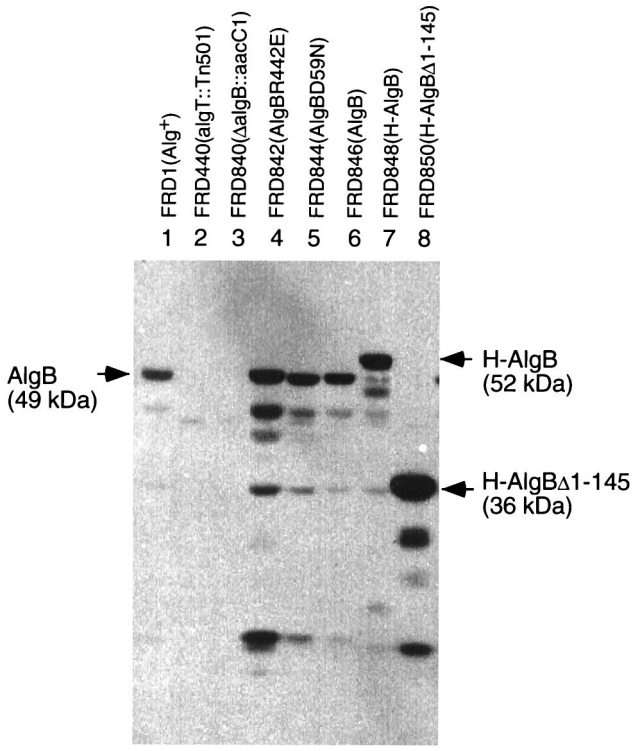

FIG. 6. Immunoblot detection of AlgB in extracts derived from *P. aeruginosa* strains. Extracts were prepared from each strain (see Materials and Methods), and 35 μg was applied in each lane. The immunoblot was performed with a 1/20,000 dilution of rabbit anti-AlgB serum. Antigen-antibody complexes were detected with enhanced chemiluminescence reagents (Amersham). Positions (and molecular masses) of H-AlgB and wild-type AlgB proteins are indicated.

The colony morphologies of these strains were examined on L agar following incubation overnight at 37°C (Fig. 7). The Δ*algB*::Ω*aacC1* mutant FRD840 had an Alg^- phenotype as expected, because *algB* is required for high-level alginate production (22). Replacing the Δ*algB*::Ω*aacC1* marker with wild-type *algB* restored the Alg^+ phenotype in FRD846. However, FRD844 (AlgB.D59N) and FRD850 (AlgBΔ1-145) also had an Alg^+ phenotype similar to that of FRD1, indicating that AlgB can promote high-level alginate production without phosphorylation. In contrast, the *algB*48 mutant FRD842, which produced AlgB.R442E with an altered DNA binding domain, was Alg^- (Fig. 7). A plasmid-borne *algB*48 allele also failed to complement an *algB*::Tn*501* mutant in *trans*. This suggests that AlgB functions as a DNA binding protein in its role in alginate production. The alginate levels produced by these strains in L broth (after 22 h of incubation at 37°C) was determined, and the values obtained were consistent with their colony morphologies; Alg^- FRD840 ($AlgB^-$) and FRD842 (AlgB.R442E) cultures synthesized only about 3% of the alginate made by the parental strain FRD1, whereas Alg^+ FRD846 ($AlgB^+$), FRD844 (AlgB.D59N), and FRD848 (H-AlgB) produced alginate levels that were similar to that of FRD1 (Fig. 7). Even FRD850, in which the entire phosphorylation domain of AlgB was deleted, still produced about 45% of wild-type alginate levels (Fig. 7). Since the mutant AlgB proteins in FRD844 and FRD850 could not be phosphorylated in vitro, these results suggest that AlgB functions in a phosphorylation-independent, DNA-binding-dependent manner to promote alginate production in mucoid *P. aeruginosa.*

A mutation in the predicted phosphorylation sites of AlgR did not affect alginate production in mucoid *P. aeruginosa*. Prior in vitro studies showed that the alginate response regulator AlgR could be phosphorylated by the enteric chemotaxis histidine protein kinase CheA and the small phosphodonor molecule acetyl phosphate (12). Similarly to AlgB and other well-characterized response regulators, AlgR contains a conserved aspartate residue (D54) which is likely the site of phosphorylation. This is supported by prior studies which demonstrated that the phosphorylated form of AlgR had biochemical properties characteristic of response regulators phosphorylated at aspartate side chains (12). More recent studies which identified *fimS*, an atypical sensor located adjacent to *algR* in *P. aeruginosa*, suggested that Asp85 might represent a second phosphorylation site unique to the AlgR subfamily of response regulators (62). To address whether phosphorylation of AlgR is required for alginate production, Asp54 and Asp85 were individually changed to asparagine residues (AlgR.D54N and AlgR.D85N). In addition, an *algR* allele expressing both alterations (AlgR.D54N.D85N) was generated. These mutations were introduced into the FRD1 chromosomal background via allelic exchange as described above (except that the Δ*algR*::Ω*aacC1* strain FRD831 was used as an intermediate), so that the *algR* alleles were in single copy under native control. In a control gene replacement experiment, wild-type *algR* restored alginate production to the FRD831 intermediate strain, forming FRD833. Interestingly, strains with mutant *algR* alleles, expressing AlgR with substitutions in the predicted sites of AlgR phosphorylation, were not affected in alginate production levels (Table 3). Strains FRD836 encoding AlgR.D54N, FRD838 encoding AlgR.D85N, and FRD839 encoding AlgR.D54N.D85N also synthesized wild-type levels of alginate (Table 3). Similarly to the results with AlgB, these data suggest that phosphorylation of AlgR does not appear to be required for activation of *algD* and alginate synthesis in mucoid *P. aeruginosa.*

DISCUSSION

In this study, we have examined the requirement for phosphorylation of the *P. aeruginosa* AlgB and AlgR alginate response regulators. Three derivatives of AlgB (H-AlgB, H-AlgB.D59N, and H-AlgBΔ1-145) were purified and examined for in vitro phosphorylation activity with purified C-KinB. The results indicated that although C-KinB could efficiently phosphorylate H-AlgB, no KinB-mediated phosphorylation of H-AlgB.D59N or H-AlgBΔ1-145 was observed under any conditions. The inability of H-AlgB.D59N to undergo phosphorylation by C-KinB was apparently not due to a lack of interaction between these two proteins, since H-AlgB.D59N (but not H-AlgBΔ1-145) was able to compete with wild-type H-AlgB in an in vitro phosphorylation assay. To evaluate the in vivo requirement for AlgB phosphorylation, a *kinB* mutant strain (FRD1049) was generated. Surprisingly, FRD1049 exhibited a mucoid phenotype and produced amounts of alginate similar to those of the parental strain FRD1. Since FRD1049 contained a null *kinB* allele, it was highly unlikely that AlgB activity in this strain was due to phosphorylation by KinB. However, AlgB could have been phosphorylated by another histidine kinase via cross-talk or with small-molecular-weight phosphodonors such as acetyl phosphate (28, 35). To address this, an *algB* allele replacement strategy was developed to examine the activities of wild-type, AlgB.D59N, and AlgBΔ1-145 derivatives in vivo. Alginate levels from strains expressing AlgB.D59N were similar to amounts produced from wild-type FRD1, and strains containing the *algB*23 allele encoding AlgBΔ1-

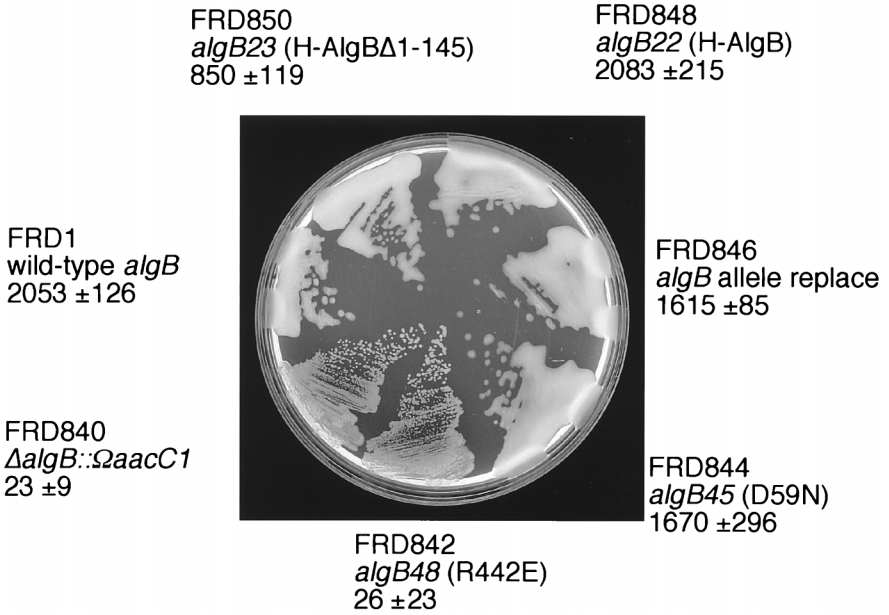

FIG. 7. Plate phenotypes of *P. aeruginosa* strains carrying wild-type or mutant *algB* alleles. The strains were constructed as outlined in Materials and Methods. L agar plates with the indicated strains indicated were incubated at 37°C for 20 h. The numbers are levels of alginate (in micrograms per milliliter) produced as quantitated by the carbazole assay (19, 26). The results are the means ± standard deviations for three independent experiments. Note that the AlgB.D59N-producing strain (FRD846), which is AlgB phosphorylation defective, still produced alginate; however, the AlgB.R442E-producing strain (FRD842), with an AlgB protein defective for DNA binding, lost the alginate-producing phenotype.

145 synthesized 45% of the wild-type levels of alginate. These results imply that there is little if any requirement for AlgB phosphorylation associated with its role in alginate overproduction in mucoid *P. aeruginosa*.

Alginate synthesis requires two independent signal transduction systems involving AlgB and AlgR (65). The data described in the present study also raise the question about a requirement for AlgR phosphorylation in alginate production. Recent work which identified a gene upstream of *algR* called *fimS*, encoding an atypical sensor kinase required for twitching motility, may shed some light on this. In these studies, which utilized *P. aeruginosa* PAK strains overexpressing the alternative sigma factor AlgU (AlgT), a mutation in *fimS* did not appear to affect alginate production whereas a mutation in *algR* resulted in a substantial reduction in alginate synthesis (62). These observations indicate that FimS and AlgR have different effects on twitching motility and alginate production. Subsequent to these studies, others reported that *fimS* (designated *algZ* in these studies) played a negative regulatory role in alginate production, since inactivation of *algZ* in a *mucA2* genetic background resulted in an approximately twofold increase in alginate synthesis (68). Our studies with *algR* alleles encoding proteins with mutations in the predicted AlgR phosphorylation site(s) indicated little if any requirement for AlgR phosphorylation in alginate production. One plausible hypothesis is that FimS modulates the phosphorylation state of AlgR; the nonphosphorylated form of AlgR may be involved in alginate production, while the phosphorylated form of AlgR may play a role in other cellular functions such as twitching motility.

During signal transduction, response regulators are usually phosphorylated at a conserved aspartate residue in the receiver module. This phosphorylation results in the activation of a nonconserved output domain culminating in an adaptive response. Response regulators have been classified into two broad categories based on the mechanism by which they are activated by phosphorylation (16). In one class of response regulators (exemplified by NtrC, ArcA, OmpR, and PhoB), the receiver and output domains interact in the unphosphorylated form, and this interaction leads to inhibition of dimerization of the receiver domain. This inhibition is relieved either by phosphorylation of the input domain or by deletion of the output domain. In the second class (characterized by CheB and FixJ), interaction between the receiver and output domains results in inhibition of the output domain, and this inhibition can be reversed by either phosphorylation or removal of the input domain. In both classes of response regulators, mutations in the conserved aspartate residue within the phosphorylation domain are almost always deleterious to function, although there are notable exceptions. For example, in *Rhizobium meliloti*, transcription of nitrogen fixation genes is induced under microaerobic conditions, and this control is modulated by the response regulator FixJ and a hemoprotein kinase, FixL. When

TABLE 3. Levels of alginate production by *P. aeruginosa algR* strains

Strain	Genotype	Alginate phenotype[a]	Alginate level[b]
FRD1	Wild type	Alg^+	1,905 ± 313
FRD831	Δ*algR*::*aacC1*	Alg^-	43 ± 2
FRD833	*algR*$^+$ (by allele replacement)	Alg^+	1,607 ± 157
FRD836	*algR*7 (AlgR.D54N)	Alg^+	1,775 ± 157
FRD838	*algR*10 (AlgR.D85N)	Alg^+	2,002 ± 240
FRD839	*algR*11 (AlgR.D54N.D85N)	Alg^+	2,238 ± 324

[a] Alginate phenotypes of *P. aeruginosa* strains containing the indicated *algR* alleles were scored as mucoid (Alg^+) or nonmucoid (Alg^-) after growth for 20 h on L agar plates.

[b] Alginate production in each strain was quantitated by the carbazole assay (26) with modifications described by Franklin et al. (19) and was expressed as micrograms per milliliter of total uronic acid. The results are means ± standard deviations for three independent experiments.

a mutant FixJ protein, FixJ.D54N, was analyzed in heterologous host *E. coli*, it was able to activate transcription of *fixK* at levels similar to those of wild-type FixJ, and this activation required FixL (45). FixL stimulation of FixJ.D54N activity was due to phosphorylation of an alternate FixJ residue (45). Phosphorylation at alternate residues in other response regulators such as CheY and OmpR has also been observed (5, 9), albeit the efficiencies of these phosphorylation reactions are much lower than those observed for the wild-type proteins. Although alternate phosphorylation of AlgB.D59N cannot be ruled out with the present data, two lines of evidence indicate that alternate phosphorylation is not likely to be the reason why AlgB.D59N retains wild-type activity in promoting alginate synthesis. First, despite numerous attempts, in vitro phosphorylation of H-AlgB.D59N or H-AlgBΔ1-145 was never observed. Second, in the cases in which alternate phosphorylation of response regulators has been demonstrated, this phosphorylation was confined to the highly conserved amino-terminal phosphorylation domain (5, 9, 45). However, FRD850 cells which expressed an AlgB derivative lacking the amino-terminal phosphorylation domain (H-AlgBΔ1-145) had a mucoid phenotype and synthesized high levels of alginate. Although it remains a possibility that the activity of H-AlgBΔ1-145 could be due to relief of the inhibitory effect imposed by the phosphorylation domain as observed in FixJ, it is more difficult to reconcile the in vivo activity of H-AlgB.D59N via this mechanism.

In *Bacillus subtilis*, the DegS-DegU two-component system controls the expression of a wide variety of genes that encode degradative enzymes and late-competence proteins (39). In this system, phosphorylated DegU was shown to be required for the expression of genes encoding degradative enzymes, as well as *degQ*, *degR*, and *srfA*, whereas nonphosphorylated DegU was capable of activating the late-competence genes *comC* and *comG* (39). Expression of genes encoding degradative enzymes was abolished in *B. subtilis* mutants which synthesized a DegU derivative that could not be phosphorylated (DegU.D56N), whereas the competence pathway was not affected. Thus, phosphorylation of the DegU response regulator apparently acts as a molecular switch controlling the expression of either the degradative-enzyme or late-competence gene. By analogy with the DegS-DegU two-component system, the KinB-AlgB pair may also have dual function in *P. aeruginosa*, whereby nonphosphorylated AlgB is required for alginate production but the phosphorylated form has another unidentified role(s) in the cell. This is supported by the observation that *algB* is expressed at low but clearly detectable levels in nonmucoid strains (64).

Strains of *P. aeruginosa* recovered from CF patients with chronic lung infections are mucoid and synthesize copious amounts of alginate. The molecular mechanism underlying overproduction of alginate in these strains has been elucidated and suggests that the activity of the alternative sigma factor σ^{22} is negatively controlled by accessory elements encoded by adjacent *muc* genes (20, 23, 31, 32, 34, 50, 66). Whereas the activity of σ^{22} in wild-type *P. aeruginosa* strains appears to be modulated by the anti-sigma factors MucA and MucB, most CF isolates including FRD1 used in our study harbor mutations in *mucA* and synthesize high levels of active σ^{22}. The levels of expression of *algB* and *algR* have been shown to be increased in mucoid strains, and this activation requires σ^{22} (64, 65) (Fig. 6 [compare lanes 1 and 2]). An attractive hypothesis to explain a lack of requirement for KinB or AlgB and AlgR phosphorylation in the control of alginate synthesis is that elevated levels of these response regulators in the cell may bypass the need for phosphorylation. If phosphorylation controls an equilibrium between active and inactive response regulators, overexpression of AlgB.D59N or AlgR.D54N or wild-type AlgB in a *kinB* mutant may lead to levels of active protein which are sufficient to promote alginate synthesis. This mechanism was proposed to account for the observation that overexpression of the *P. aeruginosa* response regulator PilR in the absence of the PilS sensor allowed for transcription of *pilA* (6). The ComA (response regulator) and ComP (sensor) proteins control competence in *B. subtilis*. Overexpression of *comA* can overcome mutations in *comP*, restoring ComA activity which is insensitive to environmental signals (14). Another example is the UhpA protein of *E. coli*, which is a response regulator required for the transcription of *uphT*, encoding the sugar phosphate transport system. UhpA activity is modulated by two membrane proteins, UhpB and UhpC. Overexpression of wild-type UhpA in a *uhpBC* mutant or high-level expression of a UhpA.D54N variant leads to wild-type activation of *uhpT*. This was not observed when *uhpA* was in single copy (60, 61). At the onset of sporulation in *B. subtilis*, levels of the response regulator Spo0A increase significantly. It has been proposed that the increase of Spo0A alone, independently of phosphorylation, mediates some regulatory interactions between Spo0A and selected promoters with high-affinity Spo0A binding sites (2). It is possible that constitutive AlgB and AlgR activities in FRD1 may be due to "runaway" σ^{22} synthesis which results in elevated levels of these response regulators. This apparently novel natural mechanism, which may represent an adaptive response to allow *P. aeruginosa* to survive in the CF lung environment, will be addressed in future studies.

The CF lung represents a unique environment for *P. aeruginosa*. Under strong selective pressure, an accumulation of mutations such as those in *mucA* occurs, leading to breakdown of the regulatory circuit of σ^{22}. It is not entirely clear what specific function of σ^{22} is selected for in the CF lung, since σ^{22} is involved in regulating alginate production, twitching motility, stress response, heat shock, and likely other unknown cellular processes (23, 49, 62, 67). Nevertheless, studies have suggested that alginate provides *P. aeruginosa* with a selective advantage in the CF lung (see reference 23 and references therein). It is possible that there are signals present in the CF lung that promote low-level alginate production and that, under these conditions, sensor proteins such as KinB and FimS are required for phosphorylation of AlgB and AlgR, respectively. While this possibility remains to be investigated, removing or blocking such signals and/or inhibiting the KinB-AlgB or FimS-AlgR signal-transducing pathways may prevent low-level alginate production by the initially colonizing *P. aeruginosa* strains. Since *P. aeruginosa* is a ubiquitous organism commonly found in soil and water, it is likely that the AlgB and AlgR signal transduction systems evolved to monitor conditions in these environments rather than in the CF lung. The development of *algB* and *algR* alleles defective in the signal transduction pathways will also allow us to investigate the natural roles of these response regulators and specific environmental cues in the production of alginate and other properties associated with *P. aeruginosa.*

ACKNOWLEDGMENTS

We thank I. Blomfield and B. Bourret for helpful discussions. We acknowledge H Schweizer for advice about allele replacement experiments and for providing us with pEX100T and pUCGM.

This work was supported by Public Health Service grants AI-35177 (D.J.W.) and AI-19146 (D.E.O.) from the National Institutes of Allergy and Infectious Diseases and in part by a Forsyth County United Way NIH grant RR-0504 (D.J.W.) and Veterans Administration Medical Research funds (D.E.O.). Oligonucleotide synthesis and amino-

terminal analyses were performed in the DNA and Protein Synthesis Core Laboratories of the Cancer Center of Wake Forest University, which is supported in part by NIH grant CA-12197.

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