Identification of the Histidine Protein Kinase KinB in *Pseudomonas aeruginosa* and Its Phosphorylation of the Alginate Regulator AlgB*†

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The exopolysaccharide alginate is an important virulence factor in chronic lung infections caused by the bacterium *Pseudomonas aeruginosa*. Two positive activators for alginate synthesis, AlgB and AlgR, are members of a superfamily of response regulators of the two-component regulatory system. AlgB belongs to the NtrC subfamily of response regulators and is required for high-level production of alginate. In this study, an open reading frame encoding a polypeptide of 66 kDa, designated *kinB*, was identified immediately downstream of *algB*. The sequence of *kinB* is homologous to the histidine protein kinase members of two-component regulatory systems. Western blot analysis of a *P. aeruginosa* strain carrying a *kinB-lacZ* protein fusion and studies of *kinB-phoA* fusions indicate that KinB localizes to the inner membrane and has a NH$_2$-terminal periplasmic domain. A KinB derivative containing the COOH terminus of *kinB* was identified immediately downstream of *algR*. Two positive activators for alginate synthesis, AlgB and AlgR, are members of a superfamily of response regulators of the two-component regulatory system. AlgB belongs to the NtrC subfamily of response regulators and is required for high-level production of alginate. In this study, an open reading frame encoding a polypeptide of 66 kDa, designated *kinB*, was identified immediately downstream of *algB*. The sequence of *kinB* is homologous to the histidine protein kinase members of two-component regulatory systems. Western blot analysis of a *P. aeruginosa* strain carrying a *kinB-lacZ* protein fusion and studies of *kinB-phoA* fusions indicate that KinB localizes to the inner membrane and has a NH$_2$-terminal periplasmic domain. A KinB derivative containing the COOH terminus of *kinB* was generated and purified. In the presence of [Y-$^{32}$PIATP], the purified COOH-terminal KinB protein was observed to undergo progressive autophosphorylation *in vitro*. Moreover, the phosphoryl label of KinB could be rapidly transferred to purified AlgB. Substitutions of the residues conserved among histidine protein kinases abolished KinB autophosphorylation. These results provide evidence that *kinB* encodes the AlgB cognate histidine protein kinase.

Chronic pulmonary infection with the bacterium *Pseudomonas aeruginosa* is a major factor in the poor prognosis and high mortality rate of patients with cystic fibrosis (CF)†. Most *P. aeruginosa* strains isolated from the CF respiratory tract overproduce an exopolysaccharide called alginate, which gives the colonies a mucoid morphology (2). This highly viscous polysaccharide plays a role in the pathogenesis of *P. aeruginosa* by imparting antimicrobial properties (3) and an adherence mechanism (4). Most of the genes involved in alginate biosynthesis are in a tightly regulated operon at 34 min on the 75-min chromosome (5). High expression of the alginate biosynthetic genes requires the activation of an alternative sigma factor (σ$^B$) encoded by *algT* (*algU*) at about 68 min on the chromosome (for review, see Ref. 6). In addition, a cascade of several positive regulators are also required for high expression of alginate genes (7, 8). Two of these, AlgB and AlgR (AlgR1), belong to the superfamily of response regulators of prokaryotic two-component regulatory systems (9, 10).

Two-component regulation is a mechanism for signal transduction to control cellular adaptations in response to environmental or physiological changes (for review, see Ref. 11). Observed in many bacterial species (12, 13), as well as in yeasts (14) and plants (15), two-component systems generally include a histidine protein kinase and a cognate regulator protein. In general, the histidine protein kinase senses a specific environmental stimulus and undergoes autophosphorylation at a histidine residue present in a highly conserved carboxyl-terminal domain of the protein. This phosphate group is subsequently transferred to an aspartate residue in the amino terminus of the response regulator, resulting in a change in the activity of the response regulator that leads to an adaptive response (11, 12). Response regulators can also catalyze kinase-independent phosphorylation and dephosphorylation by low-molecular weight phosphorylated compounds (e.g. acetyl phosphate, carbamyl phosphate, etc.), which may serve to integrate environmental control with the physiological status of the cell (16).

Alginate overproduction by *P. aeruginosa* is generally seen in strains causing pulmonary infection of CF patients. Specific signals present in the environment of the CF lung (e.g. dehydration, high osmolarity, limiting nutrients, antibiotics) may play a role in stimulating alginate production (for review, see Ref. 17). However, the role or requirement for any particular *in vivo* signal in the expression of alginate genes has not been well established. The discovery of two-component response regulators (i.e. AlgB and AlgR) suggests that environmental signals may play a role in the regulation of alginate production. Moreover, inhibitors of the two-component regulatory pathway inhibit the expression of alginate biosynthetic genes (18). However, proteins in *P. aeruginosa* with sensor kinase activity that can phosphorylate AlgB or AlgR have not been demonstrated. A gene adjacent to *algR* was recently identified that encodes a protein (FimS, AlgZ) with homology to an atypical two-component sensor (19, 20), but whether it functions as a kinase of AlgR is unknown. In this study we identified a gene called *kinB*, located immediately downstream of *algB*, that encodes a protein with high similarity to typical histidine protein kinases of two component systems. Our data indicate that KinB is an inner membrane protein with histidine protein kinase activity that is capable of promoting autophosphorylation and rapid transfer of the phosphate to AlgB.

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‡ The abbreviations used are: CF, cystic fibrosis; kb, kilobase pairs; PAGE, polyacrylamide gel electrophoresis; BisTris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxymethyl)propane-1,3-diol; ORF, open reading frame.
EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The P. aeruginosa strains utilized in this study were FRD1, an alginate-overproducing (Alg⁺) CF isolate and its derivative FRD444 (Alg⁻, AlgB::Tn501), which contains a mercury resistance (Hg²⁺) transposon marker in algB (21). Escherichia coli strains HB101 and JM109 were used in routine cloning manipulations (22); BL21(DE3) was used to express His-tagged KinB; XL-2 Blue was used to overexpress AlgB. L broth (10 g of tryptone (Difco), 5.0 g of yeast extract (Difco), 5.0 g of NaCl/liter, pH 7.5) was used for the routine culture of P. aeruginosa and E. coli. A 1:1 mixture of Pseudomonas isolation agar (Difco) and L agar was used to select for P. aeruginosa following triparental matings. Selective antibiotics used for P. aeruginosa were carbenicillin at 300 µg/ml and tetracycline at 100 µg/ml; selective antibiotics used for E. coli were ampicillin at 100 µg/ml, kanamycin at 35 µg/ml, and tetracycline at 15 µg/ml. HgCl₂ was used at 18 µg/ml both for P. aeruginosa and E. coli.

Nucleic Acid Manipulations and Plasmids—Cloned DNA fragments utilized in this study are shown in Fig. 1. Most routine genetic manipulations were performed as described elsewhere (22). Plasmid DNA was isolated from E. coli using Qian columns and procedures (Qiangen Corp.). Genomic DNA of P. aeruginosa was prepared using a protocol previously described (21). Restriction endonucleases were purchased from Boehringer Mannheim and New England Biolabs. To isolate DNA that included sequences located downstream of algB, chromosomal DNA from FRD444 (algB::Tn501) was digested with BamHI (where Tn501 is not cut by BamHI), ligated into cosmid vector pEM2R (23), packaged in vitro into λ particles (Gigapack II cloning kit, Stratagene), and transduced into HB101. One representative clone (pDJW10, Fig. 1) was isolated, and the chromosomal DNA fragment contained approximately 15-kb DNA and 10-kb DNA downstream of algB. Plasmid pDJW130 (Fig. 1) had a 0.8-kb XhoI-EcoRI fragment from pG12 (24) cloned into vector pKSI(−) that was used as a hybridization probe; it was digoxigenin-labeled by the polymerase chain reaction using T3 and T7 primers. This probe was used to identify a 4-kb ClaI-HindIII fragment from pDJW10 which contained a portion of algB and the entire kinB gene (below), which was then cloned into pSM67 (Fig. 1).

DNA Sequencing and Analysis—To prepare the DNA downstream of algB for sequence analysis, the 4-kb ClaI-HindIII fragment of pSM67 was digested with PstI or partially digested with Sau3AI, and the resulting fragments were cloned into the PstI or the BamHI site of M13mp19 (New England Biolabs), respectively. Single-stranded DNA templates were prepared from these M13mp19 clones using a sample preparation protocol (Applied Biosystems). DNA sequencing reactions were performed with a T7 DNA polymerase terminator cycle sequencing kit (Applied Biosystems) using a Perkin-Elmer DNA thermal cycler and run on an Applied Biosystems 373A DNA sequencer. DNA fragments were sequenced on both strands, and the sequence data obtained were aligned using SeqMan software (DNASTAR) on an Apple Macintosh computer. To verify alignment of the sequence contigs, a six-sequenced alignment was performed by manual sequence analysis of pSM67 (Fig. 1). The 4-kb ClaI/HindIII fragment of pSM67 was subcloned into the EcoRI fragment from M13mp19 (Fig. 1) to form pSM111, the 1.6-kb fragment containing KinB was cloned into pEcoRI restriction fragment containing "algB-kinB" to a 2.6-kb Smal/I-XbaI fragment containing phoA from pH70 (29), and cloned into pSM111, replacing the existing M1u-I-XbaI fragment, to form pSM126 and pSM127, respectively. A KinB-PhoA fusion with a junction at residue P579 was constructed by using a linker to join a M1u-I-EcoRI restriction fragment containing "algB-kinB" to a 2.6-kb Smal/I-XbaI fragment containing phoA from pH70; this was cloned into pSM111, replacing the existing M1u-I-XbaI fragment, to form pSM128 (Fig. 1). Protein fusions containing PhoA (alkaline phosphatase) were verified by Western blot analysis using rabbit anti-alkaline phosphatase (Sigma). Proteins containing PhoA fusions with alkaline phosphatase activity (i.e., localized to the periplasm) were screened for blue color on L agar containing 5-bromo-4-chloro-3-indolyl phosphate at 40 µg/ml.

Purification of the COOH Terminal of KinB (C-KinB)—To construct plasmids that overexpressed a His₆-tagged carboxy-terminal (Gly-198 to Val-595) fragment of KinB (HC-KinB), a 1.6-kb Ascl fragment of pSM67 (Fig. 1) was cloned into pNEB193 (New England Biolabs) to form pSM112, this was subsequently digested with ClaI and HindIII, and the 1.6-kb fragment containing kinB was cloned in pET29b+ (Novagen), resulting in pSM95 (Fig. 1). E. coli BL21(DE3) harboring pSM95 was agitated overnight at 37 °C in 100 ml of L broth with kanamycin. The cells were harvested by centrifugation and resuspended in 100 ml of L broth with kanamycin and 1 mM isoprropyl β-D-thiogalactopyranoside for induction of the tac promoter (Pₜac). After incubation at 30 °C with shaking for 2 h, the cells were harvested and in 20 ml of 20 mM Tris-HCl, pH 8.0, containing lysozyme (100 mg/ml) for 30 min at 4 °C. Following sonication, the lysate was centrifuged at 12,000 × g for 15 min, and the supernatant was filtered (0.45-µm disc filter, Millipore). HC-KinB in the cell extract was purified on a 2.5-ml His-Bind nickel column (Novagen) according to manufacturer's protocol. As estimated by SDS-PAGE and Coomassie Blue staining, HC-KinB was over 95% pure. The His₆ tag was removed from HC-KinB by digestion with 25 units/ml thrombin (Novagen) for 2 h at 22 °C to form C-KinB, which was subjected to an amino-terminal sequence analysis (Biology Technology Center, St. Jude Children's Research Hospital, Memphis, TN).

Production of Altered C-KinB Proteins—To obtain C-KinB proteins with substitutions in conserved residues, the Soc1-HindIII fragment in pSM95 (Fig. 1) was cloned into pALTER1 (Promega) and mutagenized using the altered sites mutagenesis system (Promega) according to the procedure suggested by the manufacturer. The mutagenic primers used were: E257Q (5' TCGTTCGCTCAAGAACTCTGCGACG 3'), H385K (5' CGCGGCGAGCGCGCGCC 3'), N504Q (5' CGCGACTGCTCTCGCGGCGGC 3'), N504D (5' CGCGACTGCTCTCGCGGCGGC 3'), and N504A (5' CGCGACTGCTCTCGCGGCGGC 3'). Following the confirmation by DNA sequence analysis, the Soc1-HindIII fragment of each kinB allele was cloned into pET28b, and mutant C-KinB proteins were expressed and purified as described above.

Purification of AlgB—E. coli XL21 Blue harboring pDJW52 (Fig. 1)
expresses algB under the control of Ptac as described previously (9). Cells from 400 ml of overnight culture of XLI21 Blue (pDJSW52) were resuspended and agitated in 400 ml of fresh L broth with ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside at 30 °C for 3 h. Cells were harvested and then incubated for 30 min at 4 °C in 20 ml of 20 mM Tris-HCl, pH 8.0, containing lysosyme (100 µg/ml). Following sonication, the lysate was centrifuged at 12,000 × g for 10 min, and the supernatant was centrifuged at 200,000 × g for 60 min. Proteins in the clear supernatant were precipitated with 35% ammonium sulfate (J. T. Baker Inc.), and the precipitant was resuspended and dialyzed against 15 mM BisTris propane, pH 7.0, 20 mM NaCl. A sample (20 ml containing 8.4 mg of protein) was loaded onto an AP-2 column (Waters) packed with Protein-PAK DEAE 40HR anion exchange matrix (Waters), and a linear 20–160 mM NaCl gradient in 15 mM BisTris propane, pH 7.0, was used to elute proteins from the column. AlgB eluted at 130 mM NaCl and was estimated by SDS-PAGE and Coomassie Blue staining to be >90% pure.

In Vitro Phosphorylation Assays—Autophosphorylation of C-KinB was performed at 22 °C in P buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl₂). C-KinB was diluted to a final concentration of 2.5 µM and distributed in 9-µl aliquots for each reaction. Each reaction was started by adding [γ-32P]ATP (30 Ci/mmol, Amersham) to a final concentration of 33.3 µM and was stopped by the addition of 3 µl of 5 X SDS sample buffer. Unincorporated label was removed by passage through a 1-ml Sephadex G-25 (Pharmacia Biotech Inc.) column, and samples were electrophoresed on a 10% polyacrylamide gel and examined by autoradiography. To examine the time course of C-KinB autophosphorylation, phosphorylation reactions were stopped by adding 10 µl of 200 mM sodium acetate, pH 4.0, and immediately spotting the mixture onto a phosphocellulose membrane (Beckman) pre-equilibrated with 25 mM sodium acetate, pH 4.0. The membranes were washed three times for 10 min each in 800 ml of buffer containing 25 mM sodium acetate, pH 4.0, and the radioactivity on the dried membranes was measured (TLCARB 2000 liquid scintillation analyzer). In studies demonstrating the transfer of phosphoseryl label from C-KinB to AlgB, 13 pmol of KinB was phosphorylated for 60 min at 22 °C in a 10-µl mixture under the conditions described above. AlgB (40 pmol) was added to the mixture, and the reaction was terminated after 90 s by adding 3 µl of 5 X SDS sample buffer. The samples were passed through a 1-ml Sephadex G-25 column to remove unincorporated label and analyzed by SDS-10% PAGE, followed by autoradiography.

Nucleotide Sequence Accession Number—The nucleotide sequence data and inferred amino acid sequence reported here for kinB have been deposited in the GenBank™ data base under accession number U97063.

RESULTS

Cloning and Identification of kinB—We examined whether a gene (kinB) encoding a sensor kinase was closely linked to a known gene (algB) encoding a response regulator that controls the alginate biosynthetic operon in P. aeruginosa. Several studies on bacterial two-component regulatory systems have shown that genes encoding a response regulator and its cognate histidine protein kinase are often linked (12). A 25-kb BamHI fragment containing the DNA flanking algB (pDJWA10, Fig. 1) was obtained from genomic DNA of P. aeruginosa PDR444, a strain with an algB::Tn501 allele (21) that provided a selectable marker (mercury resistance) for the DNA in this region. A 4-kb ClaI-HindIII fragment was then subcloned from the region immediately downstream of algB (pSM67, Fig. 1). This was subjected to a sequence analysis, and the putative kinB ORF of 1,785 bp was observed in the same direction of transcription as algB (Fig. 2). The kinB ORF had a translation initiation codon that overlapped with the algB termination codon, suggesting that expression of algB and kinB may be translationally coupled. The kinB ORF predicted a polypeptide of 595 amino acids with a molecular weight of 66,078. Two hydrophobic domains at the amino terminus of KinB were observed (underlined in Fig. 2). An 11-base pair inverted repeat sequence was located 75 bp downstream of the kinB ORF that may serve as a factor-independent terminator (shown as hatched lines in Fig. 2).

The kinB Gene Encodes a Protein with Homology to Histidine Protein Kinases—A homology search showed that the KinB sequence was similar to a number of histidine protein kinases in two-component regulatory systems. Fig. 3 depicts an alignment of kinB sequences with that of PhoR, a similar sized histidine protein kinase in Bacillus subtilis (30). Overall, KinB shows 31% identity and 59% similarity with PhoR. The most conserved sequences were in four regions that are characteristic of histidine protein kinases (marked with hatched boxes in Fig. 3). The H box is proposed to be the phosphorylation domain and may also be involved in the dimerization of the kinase monomers; the N, D/F, and G boxes are proposed to form a nucleotide binding surface in the tertiary structure within the active site (31). The residues in these boxes that are believed to be critical (marked with triangles in Fig. 3) were all conserved in KinB and PhoR. In addition, both KinB and PhoR contained two hydrophobic domains that were similarly positioned in their amino termini (underlined and overlined in Fig. 5). Both hydrophobic regions of KinB were sufficient in length to form transmembrane domains, suggesting that KinB may be localized to the inner membrane, as is PhoR.

Membrane Localization of KinB-LacZ in P. aeruginosa—A KinB-LacZ fusion protein (encoded by pSM82, Fig. 1) was constructed to test the expression of the kinB ORF in P. aeruginosa. The KinB-LacZ was predicted to be a 157.4-kDa protein, but the amino-terminal 379 amino acids of KinB (41.6-kDa) was fused to a lacZ derivative expressing all but the first eight amino acids of LacZ (115.8 kDa). As a control, an AlgB-LacZ fusion protein of 151.4 kDa was constructed (encoded by

![FIG. 1. Plasmids utilized in this study.](image)

The cloned fragment of P. aeruginosa DNA in each plasmid is depicted. The name for each is shown on the left with name of the vector shown below it in parentheses.
pSM35, Fig. 1). The plasmids containing the \textit{kinB-lacZ} and \textit{algB-lacZ} fusion genes were in suicide vectors, and their mobilization to \textit{P. aeruginosa} resulted in chromosomal integration at the site of DNA homology (Fig. 4). FRD1::pSM82 and FRD1::pSM35, harboring the respective \textit{kinB-lacZ} and \textit{algB-lacZ} fusions in single copy, both showed β-galactosidase activity, indicating that each ORF expressed a protein in \textit{P. aeruginosa}. The \textit{kinB-lacZ} and \textit{algB-lacZ} encoded fusion proteins were also analyzed in a Western blot analysis of whole cell extracts, using polyclonal antibody specific for LacZ (Fig. 4, lanes 1 and 4); this showed that their electrophoretic mobilities were consistent with the sizes predicted. The KinB-LacZ fusion produced in FRD1::pSM82 contained a large amino-terminal fragment of KinB that included both putative transmembrane domains. To test whether the KinB-LacZ hybrid localized to the membrane, whole cell extracts of FRD1::pSM82 were used to obtain fractions enriched for either cytoplasmic or membrane proteins. Extracts containing the AlgB-LacZ (FRD1::pSM35) were processed in parallel. Using anti-LacZ in the Western blot analysis, KinB-LacZ was detected in the membrane fraction, but not in the cytoplasmic fraction (Fig. 4, lane 2), suggesting that KinB was indeed associated with the membrane. In contrast, the AlgB-LacZ fusion protein, which does not contain a potential transmembrane domain (9), was detected in the cytoplasmic fraction (Fig. 4, lane 6), but not in the membrane fraction.

Study of Membrane Topology with KinB-PhoA Fusions—The two hydrophobic domains in the amino terminus (residues 13–39 and 170–190) of KinB, which may serve as transmembrane domains, were evident in the hydrophilicity plot (Fig. 5A). Thus, the region between the two putative transmembrane domains (residues 40–169) of KinB was predicted to be in the periplasmic space. To test this, KinB-PhoA fusions were constructed with junctions at residues Asp-148, Ile-211 and Phe-379 (Fig. 5B). All three KinB-PhoA fusions expressed proteins of the predicted size that were readily detected in whole cell extracts of \textit{E. coli} using a Western blot analysis and antibody specific for PhoA (Fig. 5C). In that the \textit{phoA} gene encodes the periplasmic enzyme alkaline phosphatase, such protein fusions are enzymatically active (PhoA') only if translocated to the...
Fig. 3. Alignment of P. aeruginosa KinB and B. subtilis PhoR. The sequences of two histidine protein kinases, PhoR (30) and KinB (this study), were aligned by the method of Lipman-Pearson. Numbers on the right correspond to the positions of amino acid residues in the respective polypeptide sequences. For pairwise comparisons, bars indicate identical residues, and two dots indicate amino acids with similar properties based on polarities of their side chains. Dashes in the protein sequences indicate gaps introduced to optimize alignment. Bars above the KinB sequence or under the PhoR sequence represent hydrophobic domains. Sequences in the dashed boxes represent conserved domains characteristic of histidine protein kinases. Dashes over each box indicate highly conserved or critical residues (12, 31).

Fig. 4. Membrane localization of KinB-LacZ in P. aeruginosa. A, illustration of the strategy to produce KinB-LacZ and AlgB-LacZ fusion proteins by the recombinational integration of plasmids encoding kinB-lacZ (pSM82, Fig. 1) or algB-lacZ (pSM35, Fig. 1) into the chromosome of P. aeruginosa. The vector’s bla gene, encoding carbenicillin resistance, was used for selection. B, Western blot analysis of KinB-LacZ and AlgB-LacZ fusion proteins. The proteins (30 μg) in whole cell extracts (W), and in fractions enriched for membrane proteins (M) or cytoplasmic proteins (C), were subjected to SDS-8% PAGE. Lanes 7 and 8 contained a whole cell extract of strain FRD1 (Pa) and β-galactosidase (LacZ), respectively. Proteins were transferred to a nitrocellulose membrane, and an immunostain was performed using an anti-LacZ polyclonal antibody as the probe.

periplasm (32). The KinB(D148) PhoA fusion retained the first transmembrane domain and was PhoA+ in E. coli (Fig. 4B), suggesting that the amino terminus of KinB was between the two transmembrane domains and was periplasmic. In contrast, bacteria expressing KinB(311)-PhoA and KinB(331)-PhoA, where fusions were downstream of the two transmembrane domains, were not enzymatically active for PhoA (Fig. 5B). This suggests that the COOH terminus of KinB was localized to the cytoplasm. Thus, the KinB amino terminus appears to be the periplasmic sensor domain and the C terminus contains the cytoplasmic histidine kinase domain.

Autophosphorylation of KinB—The localization of KinB to the membrane complicated the purification of native protein for studies of its potential histidine protein kinase activity. However, all of the conserved sequences for kinase activity were present in the cytoplasmic carboxyl terminus. Thus, we tested the possibility that a carboxyl-terminal fragment of KinB may be enzymatically active, as is the case for several other membrane associated sensor kinases (33–36). DNA cloning a carboxyl-terminal fragment of KinB from Gly-198 to the His tag vector pET28.b to form pSM95 (Fig. 1). This plasmid expressed a His6-tagged fusion protein (HC-KinB), which was purified using a nickel sulfate affinity column. To remove the His6 tag, the purified fusion protein was digested with thrombin, providing a His6-tagged site (also cleaved the KinB protein between residues Arg-243 and Gln-244 to generate a 39.4-kDa C-KinB polypeptide). Nevertheless, this C-KinB fragment still retained all the sequences predicted to function as a histidine protein kinase (see Fig. 3). To test this, C-KinB was incubated with [γ-32P]ATP (33 μM) showed progressive autolabeling over the 1–60 min period examined (Fig. 6A, lanes 1–6). Incubation with [γ-32P]ATP at 33 or 66 μM for 40 min showed similar labeling of C-KinB (compare lanes 5 and 7), suggesting that ATP was not a limiting factor in these reactions. Accordingly, incubation with 50 pmol of C-KinB for 40 min did show increased labeling (Fig. 6A, compare lanes 5 and 8). As a control, C-KinB (25 pmol) was incubated with [γ-32P]ATP (15 μM) for 60 min (Fig. 6A, lane 9), and no labeling was observed; this ruled out the possibility of nonspecific binding of ATP by C-KinB. The autoradiogram showing autolabeling of C-KinB suggested that the level of protein phosphorylation (i.e., the balance of autophosphorylation and dephosphorylation) was not maximum by 60 min. Thus, a quantitative time course of C-KinB
autophosphorylation was performed using liquid scintillation (Fig. 6B). This showed that the maximum level of phosphorylated C-KinB under these conditions did not reach a plateau until approximately 5 h of incubation. One possible reason for this overall slow reaction was a high rate of C-KinB dephosphorylation. However, this appeared not to be the case because the phosphoryl label on C-KinB was stable after incubation with a chase of cold ATP (333 μM) was incubated with [γ-32P]ATP (as described above) were incubated for 0–5 h and then spotted onto a phosphocellulose membrane, which was then washed to remove unincorporated label, and subjected to SDS-10% PAGE. The plot shown was based on the average of three independent experiments. C, to determine the stability of phosphorylated C-KinB, C-KinB (2.5 μM) was incubated with 33.3 μM [γ-32P]ATP at room temperature for 1 h, and 333 μM unlabeled ATP was added to each of the reaction mixtures. The reactions were terminated after 0, 2, 4, 8, 15, and 30 min, removed of unincorporated label, and subjected to SDS-10% PAGE followed by autoradiography (lanes 1–6, respectively). Incubation of C-KinB with 15 μM [α-32P]ATP for 60 min under the same conditions was performed to confirm that nonspecific ATP binding was not a factor (lane 9). B, time course of autophosphorylation of C-KinB. Samples containing C-KinB and [γ-32P]ATP (as described above) were incubated for 0–5 h and then spotted onto a phosphocellulose membrane, which was then washed to remove unincorporated label. Incorporation of [32P] into C-KinB was determined by the radioactivity (counts/min (CPM)) retained on the membranes.

C-KinB Mutants Altered at Conserved Sequences Are Affected in Autophosphorylation—We tested whether autophosphorylation activity required sequences in KinB that are homologous to those of other sensor kinases. Critical residues in histidine protein kinases that were conserved in C-KinB (described above, see Fig. 3) were altered by site-directed mutagenesis of kinB. Mutant alleles of kinB were generated that expressed the following mutant HK-CinB proteins: H385K and H385Q, in which His-385 in the H box (i.e. the predicted site of phosphorylation) was changed to Lys and Gln, respectively; N504Q, where Asn-504 in the N box was mutated to Gln; D532N and D532E, in which Asp-532 of the D/F box was changed to Asn or Gln, respectively; and G560A where Gly-560 in the G box was substituted for Ala. Mutant derivatives of HC-KinB were purified in the same manner as wild-type HC-KinB and estimated to be >95% pure by SDS-PAGE. The His6 tags on these proteins were also removed by thrombin digestion. Equivalent amounts of wild-type and mutant C-KinB derivatives, after treatment with thrombin, were examined by SDS-PAGE for relative stability of the proteins (Fig. 7A). Only C-KinB D532E (Fig. 7A, lane 7) showed any evidence of degradation beyond removal of the His6-Arc-243 peptide (despite 27 other Arg residues, the preferred site of thrombin cleavage). When each protein (2.5 μM) was incubated with [γ-32P]ATP (33 μM), the wild-type C-KinB sequence showed strong autophosphorylation activity (Fig. 7B, lane 1). However, labeling of the mutant proteins was undetectable except for the C-KinB D532N derivative in which a trace amount of phosphorylated protein was detected (Fig. 7B, lane 5). The C-KinB E257Q protein had a substitution at a nonconserved residue, and it showed autophosphorylation that was comparable with that of wild-type (Fig. 7B, lanes 8).
variant proteins were treated with thrombin to remove the His6 tag. Kinase activity. Following purification, wild-type HC-KinB and the single amino acid substitutions in residues predicted to affect histidine matographic procedures (Fig. 8A, lane 4) was not autophosphorylated when it was incubated with [γ-32P]ATP as determined by SDS-PAGE and autoradiography. Wild-type C-KinB (lane 1) became radiolabeled, and complete dephosphorylation of C-KinB also had two hydrophobic domains at the amino terminus.

Phosphotransfer from C-KinB to AlgB—To determine whether AlgB-KinB may function as a two-component regulatory system, the ability of phosphorylated C-KinB to donate a phosphate group to AlgB was examined. AlgB was overexpressed in E. coli and purified (>90%) using standard chromatographic procedures (Fig. 8A, lane 4). Purified AlgB alone was not autophosphorylated when incubated with [γ-32P]ATP as determined by SDS-PAGE and autoradiography (Fig. 8B, lane 1). As shown above, purified C-KinB (1.3 μM) incubated with [γ-32P]ATP for 60 min showed autophosphorylation (Fig. 8B, lane 2). However, when AlgB (40 pmol) was incubated for 90 s with autophosphorylated C-KinB (K*), AlgB became radiolabeled, and complete dephosphorylation of C-KinB was also observed (Fig. 8B, lane 4). Other studies of response regulators (e.g., CheY) indicate that Mg2+ is required for phosphorylation (37). This also appears to be the case with AlgB, since no AlgB phosphorylation was observed when the protein was preincubated with EDTA to chelate divalent cations (Fig. 8B, lane 3). In other experiments, maximum phosphotransfer from 32P-C-KinB to AlgB was observed after only 20–40 s of incubation (data not shown). Taken together, the above results show that KinB in P. aeruginosa is a member of the sensor kinase superfamily with histidine kinase activity.

DISCUSSION

The genes involved in alginate biosynthesis are under complex control by a cascade of regulators (6, 8). Two positive regulators of alginate production, AlgB and AlgR, affect transcriptional activation of the alginate biosynthetic operon at algD, and both have sequence similarity to the family of response regulators of two-component systems (9, 10). This suggested that the production of alginate by P. aeruginosa is influenced by environmental factors, some of which may be found in the unique environment of the CF lung (17). Prior to the recent description of FimS and its association with AlgR (19), no putative cognate sensor for AlgR had been recognized. However, FimS (also known as AlgZ) does not possess sequence similarity to typical histidine protein kinases (19, 20). The goal of this study was to identify KinB, a cognate sensor for AlgB, followed by tests for their potential interaction via phosphorylation. In that genes encoding histidine protein kinases are often closely linked to genes for their cognate response regulators (12), we examined the DNA immediately downstream of algB, and as a result kinB was discovered. KinB had a predicted molecular mass of 66 kDa and showed sequence similarity to many histidine protein kinases of two-component regulatory systems. KinB had all four conserved “boxes” characteristic of histidine protein kinases. Like many of them, KinB also had two hydrophobic domains at the amino terminus.
that are of sufficient length and hydrophobicity to span the inner membrane. These observations led to an analysis of a KinB-LacZ fusion protein in *P. aeruginosa* that suggested that KinB was indeed a membrane protein. An analysis of KinB-PhoA fusions supported the predicted membrane topology of KinB that the region between the two hydrophobic domains was in the periplasm. The COOH terminus of KinB, which contained amino acid residues conserved with other sensor kinases, was apparently localized to the cytoplasm. During appropriate *in vivo* conditions, the amino-terminal domain may act as an environmental sensor of some unknown factor(s) and transduce that information to the cytoplasmic domain to affect its kinase activity. It is difficult to speculate at this time just what environmental signal(s) might activate KinB, as its periplasmic domain has no significant similarity with any other known protein.

Most sensor kinases studied are capable of undergoing autophosphorylation at a conserved histidine residue in the H domain of the protein (38). Purified C-KinB was shown in this study to undergo progressive autophosphorylation when incubated with \(\gamma\)-\[^{32}\text{P}]\text{ATP}\). Interestingly, the level of autophosphorylated protein did not reach its maximum until about 5 h at room temperature in the presence of excess \(\gamma\)-\[^{32}\text{P}]\text{ATP}\). This rate is quite slow when compared with the autophosphorylation of other sensor proteins under similar conditions. These sensors include derivatives of ArcB (35) and EnvZ (39) that were deleted of their amino-terminal transmembrane domains, and they have been shown to reach maximum autophosphorylation within minutes. Since the phosphorylated form of C-KinB appeared quite stable, a high intrinsic phosphatase activity is not likely, and an explanation for the atypically slow autophosphorylation of C-KinB is not currently available. However, it is possible that the deletion of the amino terminus affected its autophosphorylation activity, even though C-KinB contained the entire kinase domain. The oligomeric state of many sensor kinases is important for their autophosphorylation activity (40–42). The periplasmic domain of some kinases facilitates dimerization when it is bound by environmental stimulatory ligands (43, 44). The rapid autophosphorylation seen in amino-truncated ArcB and EnvZ may be due to strong protein-protein interactions that remain between the monomers, which is suggested by the observed aggregation and precipitation of truncated ArcB and EnvZ with the membrane fraction when overexpressed in *E. coli* (35, 39). In contrast, when C-KinB was overexpressed, it remained soluble. It is currently not clear whether the native form of KinB forms a dimer or whether dimerization affects KinB autophosphorylation activity. Another explanation for the observed kinetics of C-KinB phosphorylation also relates to the soluble nature of C-KinB. When “tethered” to a membrane, as is the case for native KinB, the effective concentration of KinB may be higher than that observed with the soluble C-KinB used in these studies. In addition, the reaction condition for the C-KinB autophosphorylation assay used here may not be optimal for this protein, although similar conditions were used in the phosphorylation of truncated ArcB and EnvZ (35, 39).

Since the sequence of KinB showed high homology with other sensor kinases, substitutions of the conserved residues were made to verify that KinB is a new member of this conserved superfamily of histidine protein kinases. When the predicted histidine phosphorylation site in KinB (His-385 in the H box) was changed to either a lysine or a glutamate, autophosphorylation of C-KinB was completely lost. Moreover, mutations affecting other conserved boxes all had deleterious effects on the kinase activity, suggesting that KinB is a typical histidine protein kinase. Interestingly, while no phosphorylated protein was detected when Asp-532 in the D/F box was substituted for a glutamate, changing the same residue to an asparagine permitted some residual C-KinB autophosphorylation.

The ability of phospho-C-KinB to phosphorylate the purified response regulator AlgB was also demonstrated. When AlgB was incubated with the phosphorylated C-KinB at a molar ratio of 3 to 1, the phosphoryl group was rapidly transferred to AlgB and completed by 40 s. This rate is similar to that observed between other sensor-regulator pairs (37, 45). Also, similar to the phosphorylation of other response regulators (37, 45), AlgB phosphorylation was inhibited by EDTA, suggesting the requirement of Mg\(^{2+}\) in the phosphorylation reaction. Magnesium has been shown to bind at an aspartate-rich acid pocket within the active site of the response regulator phosphorylation domain. Binding of Mg\(^{2+}\) causes conformation changes in the response regulator, and this likely facilitates the phosphorylation reaction between histidine protein kinases and response regulators (46–49). Previous studies with the alginate response regulator AlgR demonstrated that AlgR was capable of being phosphorylated by the well-characterized histidine protein kinase CheA and by small phospho-donor molecules (50). Despite numerous attempts, AlgB could not be phosphorylated by CheA (data not shown). This suggests that phosphorylation of AlgB by C-KinB has a relatively high specificity. The possibility of AlgR phosphorylation by C-KinB, as well as the involvement of small phospho-donor molecules in AlgB phosphorylation, is currently being examined.

At least three other sensor kinase-regulator pairs have been reported in *P. aeruginosa*, but this is the first case that *in vitro* phosphorylation of the sensor and the regulator has been demonstrated in this organism. Besides AlgB-KinB, there are two other typical two-component regulatory systems: PilS-PilR are involved in the regulation of expression of type IV fimbriae (49), and PfeS-PfeR control the expression of the ferric enterobactin receptor, PfeA (51). The genes for the histidine protein kinase and the response regulator in each of these two systems are also next to each other (49, 51). The organization of pfeR-pfeS is strikingly similar to that of algB-kinB, in that the start codon for pfeS also overlaps the stop codon for pfeR (51). The three proteins, PilS, PfeS, and KinB, all have conserved residues characteristic of histidine protein kinases, but little homology beyond that. It appears likely that KinB responds to signals different from that of PilS and PfeS. Recently, another sensor-kinase pair, FimS-AlgR, has been suggested to belong to a new family of transmitter-receiver response regulators (19, 20). However, in that the predicted FimS (AlgZ) sequence lacks a conserved H box, it has been postulated that FimS may not undergo autophosphorylation, although it may still be able to transfer a phosphate group to AlgR (19). It will be of interest to determine to what extent the roles of algB-kinB system and fimS-algR system overlap in control of the virulence factors in this opportunistic pathogen.

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