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Regulation of Nucleoside Diphosphate Kinase and Secretable Virulence Factors in *Pseudomonas aeruginosa*: Roles of *algR2* and *algH*

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Alginate is an important virulence factor for *Pseudomonas aeruginosa* during infection of the lungs of cystic fibrosis patients. The genes encoding enzymes for alginate production by *P. aeruginosa* are normally silent. They are activated in response to several environmental conditions, including high osmolarity, exposure to ethanol, or long-term growth under conditions of nutrient deprivation. Several genes which participate in the activation of alginate gene promoters have been identified; among these is the *algR2* (*algQ*) gene. AlgR2 is an 18-kDa protein which has been shown to regulate the critical *algD* gene encoding GDP-mannose dehydrogenase as well as to regulate the levels of a tricarboxylic acid cycle enzyme, i.e., succinyl coenzyme A synthetase, and nucleoside diphosphate kinase (Ndk), an enzyme involved in nucleoside triphosphate synthesis. Succinyl coenzyme A synthetase and Ndk form a complex in *P. aeruginosa*. While *algR2* is required for alginate synthesis at 37°C, an *algR2* insertion mutant was still able to make alginate slowly at 37 or at 30°C. We used this observation to identify and clone a gene, termed *algH*. A strain with mutations in both *algR2* and *algH* is unable to produce alginate at either 37 or 30°C, and it is fully defective in Ndk production.

In the early stages of infection of the lungs of patients with cystic fibrosis (CF), *Pseudomonas aeruginosa* exists in its typical nonmucoid form. As the disease progresses, the infecting bacteria undergo a developmental change which results in the production of a thick, viscous exopolysaccharide capsule termed alginate. Recovery of mucoid *P. aeruginosa* from the sputum of CF patients is an indicator of a poor prognosis. The alginate capsule has been shown to reduce the effectiveness of certain antibiotics (30), to inhibit phagocytosis (1, 17), and to promote adherence to the epithelial cells of the respiratory tract (22). *P. aeruginosa* grows as a biofilm in the CF lung, which is important for the expression of alginate genes, adherence, and survival (5, 19). It has also been demonstrated that mucoid *P. aeruginosa* organisms are better able to survive in a nutritionally poor environment than their nonmucoid counterparts (36). All these features point to the alginate capsule as an important virulence factor which helps *P. aeruginosa* survive in the harsh and unique environment of the CF lung.

Alginate production is rare outside the lungs of CF patients. While environmental isolates and isolates from burns, urinary tracts, and non-CF lung conditions such as bronchitis and asthma retain the genetic capacity to produce alginate, the genes involved in alginate production are typically silent in these cases. Furthermore, the mucoid CF isolates revert to the nonmucoid form upon continued cultivation in the laboratory. This indicates that *P. aeruginosa* maintains tight control over expression of alginate genes, activating them in specific and rare environments. Studies with nonmucoid *P. aeruginosa* have shown that slowly growing cells cultivated in nutrient-poor environments (33, 35, 36) or observed during long-term growth in rat lung (31, 41) are prone to undergo transition to mucoidy. This indicates a potential role for the energy status of the cell in the conversion to mucoidy (36). Several regulatory genes which are involved in alginate production have been identified. These include the genes for response regulator-type proteins AlgR1 (AlgR) and AlgB, the histone-like protein AlgR3 (AlgP), the putative alternate sigma factor AlgU (AlgT) and putative anti-sigma factors MucA and MucB (AlgN), and AlgR2 (AlgQ) (reviewed in references 8, 20, and 22). The roles of these genes have largely centered on their effects on expression of the *algD* gene encoding GDP-mannose dehydrogenase or the *algC* gene encoding phosphomannomutase (43). *algR2* has been shown to also regulate succinyl coenzyme A synthetase (Scs), an enzyme of the tricarboxylic acid cycle, and nucleoside diphosphate kinase (Ndk), an enzyme involved in (deoxy)nucleoside triphosphate [(d)NTP] production (28). Scs and Ndk form a complex in *P. aeruginosa* (15). The expression of *algD* is also reduced in the *algR2* mutant (7, 14), but it is not known at present if the effect on *algD* is direct or indirect. In this report, we show that a second gene, *algH*, is also involved in the regulation of Ndk, as well as that of virulence factors such as alginate, siderophore, rhamnolipid surfactant, and extracellular protease production.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are shown in Table 1. For antibiotic selection of *Escherichia coli*, ampicillin was used at 75 μg/ml, tetracycline was used at 30 μg/ml, and kanamycin was used at 50 μg/ml. For selection of *P. aeruginosa*, carbenicillin was used at 300 μg/ml and tetracycline was used at 300 μg/ml on solid media and at 100 μg/ml in liquid media. An *algR2 algH* double mutant was obtained by mutagenesis of 8830R2::Cm with ethyl methanesulfonate as described by Darzins and Chakrabarty (6). The mutagenized culture was plated on *Pseudomonas* isolation agar (Difco, Detroit, Mich.), and nonmucoid mutants were selected for further characterization. Plasmids were introduced into *P. aeruginosa* by triparental mating using pRK2013 as the helper plasmid (9).

DNA manipulations. A genomic library of 8830R2::Cm was prepared as described by Darzins and Chakrabarty (6), by using genomic DNA isolated by the procedure of Goldberg and Ohman (12). Four micrograms of genomic DNA was partially digested with *Hin*dIII to yield fragments of approximately 20 kb. This DNA was ligated into 11 μg of pCP13, completely digested with *Hin*dIII, and dephosphorylated. The resulting DNA molecules were packaged into lambda phage heads with an in vitro packaging kit (Amersham, Arlington Heights, Ill.) and used to infect *E. coli* JM109. The library was introduced into

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Reference
Strains		
P. aeruginosa		
8830	*his-1 alg*$^+$	6
8830R2::Cm	*his-1 algR2*::Cm	28
8830R2::Cm*algH*	*his-1 algR2*::Cm *algH*	This study
E. coli JM109	Δ(*pro-lac*) *recA1 thi-1 supE endA gyrA96 hsdR relA1* (F′ *traD36 proAB lacI*^{q} *lacZ*Δ*M15*)	42
Plasmids		
pCP13	IncP1 Tc^r Km^r *mob*$^+$	6
pRK2013	ColE1 Tra[RK2]$^+$ Km^r	9
pMMB66EH	IncQ *lacI*^{q} *Ptac rrnB* Ap^r *mob*$^+$	11
pJK662	pMMB66EH *algR2*	14
pDS26	pCP13 *algH*, 20-kb insert	This study
pDS27	pMMB66EH *algH*, 6-kb *Pst*I insert	This study
pDS28	pMMB66EH *algH*, 3.3-kb *Eco*RI-*Pst*I fragment	This study
pDS29	pMMB66EH, 2-kb *Eco*RI-*Sal*I fragment	This study

8830R2::Cm*algH* by triparental mating, and transconjugates were screened visually for the mucoid phenotype. Subcloning of restriction fragments was performed according to standard procedures (27).

Enzyme assays. The autophosphorylation assays for Ndk and Scs were done with crude extract as described previously (28). The thin-layer chromatography assay for Ndk with and without Tween 20 has also been described previously (28). Growth curves in the presence and absence of Tween 20 were determined according to the method of Schlictman et al. (29). Siderophore was assayed as described by Venturi et al. (37). Protease in culture supernatants was assayed as described by Kubo and Imanaka (18) with either casein (10) or azocasein (3) as the substrate. Rhamnolipid biosurfactant was assayed as described by Morikawa et al. (24).

RESULTS

Isolation and characterization of an *algR2 algH* double mutant. The *algR2* gene has previously been shown to be required for alginate synthesis, and *algR2* mutants cannot produce alginate at 37°C (7, 14). When an *algR2* insertional mutant, 8830R2::Cm (28), was constructed, it was also found to be nonmucoid when grown on solid medium at 37°C after 2 days of incubation. However, upon extended incubation (>5 days), we found that even the *algR2* insertion mutant could produce small amounts of alginate. This effect was more pronounced when the mutant was grown at 30°C; mucoid colonies were visible even after overnight growth. The strain retained chloramphenicol resistance, the marker used to disrupt *algR2*. When a mucoid culture grown at 30°C was restreaked and grown at 37°C, the strain was again found to be nonmucoid after 2 days of growth, indicating that the *algR2*::Cm mutation was intact and that the mucoid phenotype seen was not due to a reversion of the *algR2*::Cm mutation. Since the *algR2* insertion mutant could make alginate under certain conditions, it indicated the presence of another gene that could supply the missing *algR2* function and restore mucoidy under those conditions.

The fact that the *algR2* mutant could produce alginate at 30°C allowed us to screen for a mutant with a defect in this gene, which we will call *algH*, by reasoning that an *algR2 algH* double mutant would be nonmucoid at both 37 and 30°C. The *algR2* mutant 8830R2::Cm was mutagenized with ethyl methanesulfonate as described in Materials and Methods, and colonies nonmucoid at 30°C were selected. Several nonmucoid isolates were obtained. These isolates would be expected to contain mutations in *algH* as well as in other alginate biosynthetic and regulatory genes in addition to the *algR2*::Cm mutation already present. To eliminate the latter, the *algR2* gene was introduced on plasmid pJK662 into the mutants and screened for complementation (mucoidy) at 37°C. We reasoned that a double mutant with mutations in the *algR2* and *algD* genes, for example, would not be complemented by *algR2*. However, a double mutation in *algR2* and *algH* might be complemented by *algR2*, provided that *algH* is not absolutely required for mucoidy at 37°C. Two such isolates, termed 8830R2::Cm*algH*35 and 8830R2::Cm*algH*48, were obtained. The 8830R2::Cm*algH*48 mutant appeared to be somewhat leaky and was discarded. The remaining isolate was termed 8830R2::Cm*algH* and used for further characterization.

Cloning of *algH*. With a mutation in the *algH* gene available, it was possible to clone the gene by looking for complementation of the *algR2 algH* double mutant to mucoidy at 30°C. A genomic library of 8830R2::Cm was constructed. This strain was used to avoid recloning the *algR2* gene. The genomic library was introduced into 8830R2::Cm*algH* by triparental mating. Transconjugates were plated on selective media (*Pseudomonas* isolation agar plus tetracycline) and scored visually for the mucoid phenotype. Approximately 80,000 colonies were screened and 15 mucoid isolates were obtained. Of these, 5 were still mucoid after being restreaked on *Pseudomonas* isolation agar supplemented with tetracycline, identifying the other 10 as unstable spontaneous revertants. Of these five isolates, cosmids from three were able to restore mucoidy to 8830R2::Cm*algH* after passage through *E. coli*. Restriction enzyme digestion patterns of the cosmids isolated from these three isolates were identical (data not shown), and it was concluded that they represented three isolates of the same cosmid. This cosmid was termed pDS26.

Restriction fragments of pDS26 were subcloned into pMMB66EH and tested for their ability to complement the *algR2 algH* double mutant as shown in Fig. 1. This procedure identified a 3.3-kb *Eco*RI-*Pst*I fragment (pDS28) capable of complementing the *algR2 algH* double mutant. We are currently in the process of defining the minimum region of the insert from pDS28 required for complementation for the purpose of DNA sequencing.

Activities of Scs and Ndk in the *algR2 algH* double mutant. The *algR2* gene has been shown to regulate the levels of Scs and Ndk (28). The levels of these two proteins are greatly reduced in the *algR2* mutant, but some residual activity does remain (approximately 10 to 20%). We wanted to determine if the *algR2 algH* double mutant showed levels of Scs and Ndk further reduced from those of the *algR2* mutant. Assaying for

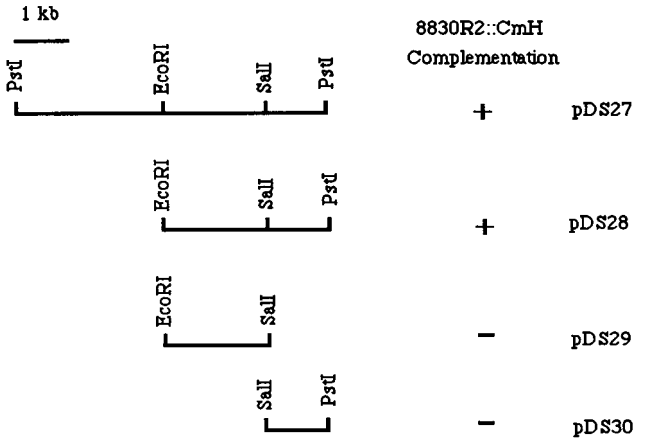

FIG. 1. Localization of the *algH* gene. The restriction fragments were subcloned into pMMB66EH and introduced into 8830R2::Cm*algH* to test for complementation (mucoidy).

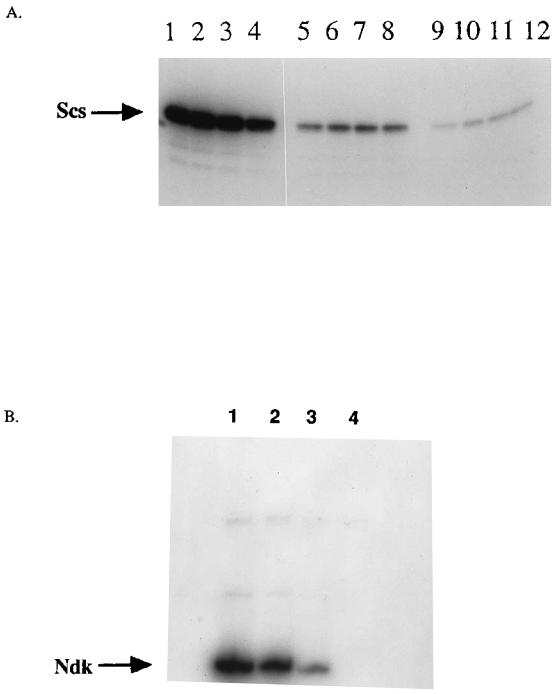

FIG. 2. (A) Effects of *algR2* and *algR2 algH* mutations on autophosphorylation of Scs. Lanes 1 to 4 contain crude extract from the parental strain 8830 used in the phosphorylation assay for 10, 30, 60, and 180 s; lanes 5 to 8 contain crude extract from 8830R2::Cm used in the phosphorylation assay for 10, 30, 60, and 180 s; and lanes 9 to 12 contain crude extract from 8830R2::Cm*algH* used in the phosphorylation assay for 10, 30, 60, and 180 s. Equal amounts of protein from the three extracts were used in the presence of [γ-^{32}P]ATP for the autophosphorylation assay. (B) Effects of *algR2* and *algR2 algH* mutations on Ndk autophosphorylation. Lane 1 contains purified Ndk from *P. aeruginosa*, lane 2 contains crude extract from the parental strain 8830, lane 3 contains crude extract from 8830R2::Cm, and lane 4 contains crude extract from 8830R2::Cm*algH*. Equal amounts of protein from crude extracts were used for lanes 2 through 4.

Scs and Ndk levels is made easy by the fact that Scs and Ndk can be detected by autophosphorylation. Extracts of 8830, 8830R2::Cm, and 8830R2::Cm*algH* were assayed by autophosphorylation in the presence of [γ-^{32}P]ATP, and the results for Scs are shown in Fig. 2A. In the *algR2* mutant, there is a significant reduction in the levels of autophosphorylated Scs, as shown previously (28). Upon introduction of a second mutation, in *algH*, the levels of phosphorylated Scs dropped even further. A similar result was obtained when Ndk autophosphorylation was examined, as shown in Fig. 2B. A mutation in *algR2* reduces the level of phosphorylated Ndk, and a mutant deficient in both *algR2* and *algH* shows essentially no phosphorylated Ndk. The fact that the reduced levels of Ndk were due to reduced amounts of Ndk protein was shown by Western blotting (immunoblotting) (data not shown). The reduced level of phosphorylated Ndk can be correlated with reduced Ndk activity, as seen in Fig. 3. Ndk can transfer the terminal phosphate from any NTP to any NDP, or to their derivatives. In the assay system used, Ndk or the sample to be tested was incubated with [γ-^{32}P]ATP and nonradioactive UDP, producing [γ-^{32}P]UTP and ADP. The [γ-^{32}P]UTP can be separated from any unreacted [γ-^{32}P]ATP by thin-layer chromatography and visualized by autoradiography. Using this assay, we have previously shown that Ndk is not the only enzyme in *P. aeruginosa* capable of performing this conversion (28, 29). In extracts of the parental strain 8830, even in the presence of neutralizing antibody against Ndk, another enzyme(s) can still produce [γ-^{32}P]UTP from UDP and [γ-^{32}P]ATP. This interfering activity can be inhibited with Tween 20 without affecting Ndk activity (28). As shown in Fig. 3, the parental strain 8830 can generate [γ-^{32}P]UTP in the presence of either Tween 20 or Ndk antibody (lanes 3 and 4), but when both are included, no activity is seen (lane 5). The *algR2* mutant showed a reduced level of Ndk activity in the presence of Tween 20 to inhibit interfering kinase(s) (Fig. 3, lane 8). The *algR2 algH* double mutant had essentially no detectable Ndk activity in the presence of Tween 20 (Fig. 3; compare lanes 3, 8, and 13). It is to be noted that purified Ndk was completely inhibited by antibody against Ndk (Fig. 3, lane 19), while Tween 20 had no effect on Ndk activity (Fig. 3, lane 18). Thus, the reduced levels of phosphorylated Ndk in the *algR2*::Cm mutant and the *algR2 algH* double mutant (Fig. 2B) correlate with reduced or essentially absent Ndk activity.

The data concerning Tween 20 inhibition of another Ndk-like activity demonstrate that at least two activities can form [γ-^{32}P]UTP from UDP and [γ-^{32}P]ATP. Considering the obvious importance of NTPs to the cell, it is not surprising that multiple activities for NTP production exist. We have shown that in the presence of Tween 20 in vitro, Ndk activity was greatly reduced in the *algR2* mutant and virtually undetectable in the *algR2 algH* double mutant (Fig. 3). However, primary control is mediated through *algR2*. Thus, two regulatory proteins (the *algR2* and *algH* proteins) control the level of Ndk in the cell. We wanted to determine if this in vitro effect of Tween 20 could be translated into an in vivo effect. To test this possibility, we grew 8830, 8830R2::Cm, and 8830R2::Cm*algH* in Luria broth with 0.1% Tween 20, since it could be expected that strains unable to produce sufficient quantities of NTPs would be affected in their growth. Growth curves are shown in Fig. 4. In the absence of Tween 20 (Fig. 4A), there was little if any difference between the strains. However, in the presence of Tween 20 (Fig. 4B), the *algR2* mutant grew significantly more slowly than its parent, 8830, and the *algR2 algH* double mutant did not grow at all. Thus, we are able to show in vitro in the presence of Tween 20 that the *algR2* and *algR2 algH* mutants are deficient in Ndk activity and that when grown in the presence of Tween 20, which inhibits the alternate kinase, the *algR2* and *algR2 algH* mutants show drastically slower growth.

Alginate, siderophore, rhamnolipid biosurfactant, and extracellular protease activities in *algR2* and *algR2 algH* mutants. As described above, the *algR2 algH* mutant is unable to

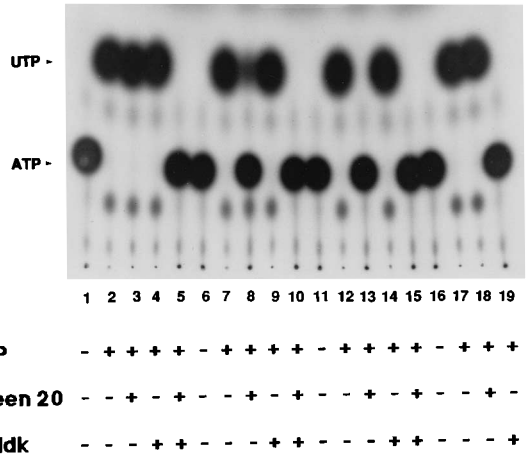

FIG. 3. Ndk activity in *algR2* and *algR2 algH* backgrounds. Lanes 1 to 5 contain crude extract from 8830, lanes 6 to 10 contain crude extract from 8830R2::Cm, lanes 11 to 15 contain crude extract from 8830R2::Cm*algH*, and lanes 16 to 19 contain purified Ndk from *P. aeruginosa*. The inclusion of UDP, 0.05% Tween 20, and/or neutralizing antibody against Ndk (α-Ndk) is indicated by a plus sign; a minus sign indicates the absence of a substance. The light spot migrating ahead of the ATP spot is [γ-^{32}P]GTP, produced from trace amounts of GDP contaminating the UDP preparation.

make alginate at 37 or 30°C, while the *algR2* mutant is mucoid at 30 but not 37°C. The ability of *algR2* or *algH* in *trans* to restore alginate synthesis was tested, and the data are shown in Table 2. It is interesting that either *algR2* or *algH* in multiple copies is able to restore alginate synthesis to the *algR2 algH* mutant.

A gene similar to *algR2*, *pfrA*, in *Pseudomonas putida* WCS358 has been described. This gene regulates the production of siderophore and is important in the regulation of the siderophore promoter in response to iron limitation (37). It was shown that a *P. aeruginosa* mutant generated by a chemically induced point mutation in *algR2*, strain 8882, is also deficient in siderophore activity, producing only 39% of the siderophore produced by the parental strain (37). Given this information, we tested the ability of the *algR2* insertion mutant and the *algR2 algH* mutant to produce siderophore; the results are shown in Table 3. The mutation in *algR2* reduced siderophore activity as expected, and a second mutation in *algH* resulted in a further reduction in activity. Complementing the *algR2* mutant or the *algR2 algH* double mutant with *algR2* restored activity.

Both *algR2* and *algH* appear to regulate the production of alginate and siderophore, two important virulence factors. To determine if other secreted products were controlled by *algR2* or *algH*, we assayed for extracellular protease production in the supernatants of parental and mutant cultures. The results of the protease assays are shown in Table 4. Protease activity does appear to be affected by *algR2*, but instead of showing a lower level of protease activity, as was the case with alginate and siderophore production, the *algR2* mutant showed a higher level. Complementation of the *algR2* mutant or the *algR2 algH* double mutant with *algR2* reduced the level of protease production to parental levels.

Given that three secreted factors, i.e., alginate, siderophore, and protease, appear to be influenced by *algR2* and *algH*, we decided to examine the effects of *algR2* and *algR2 algH* mutations on another secreted product, rhamnolipid biosurfactant. Biosurfactants are surface-active compounds important in emulsification of hydrophobic substances, and the *P. aeruginosa* rhamnolipid has been shown to be an effective emulsifier

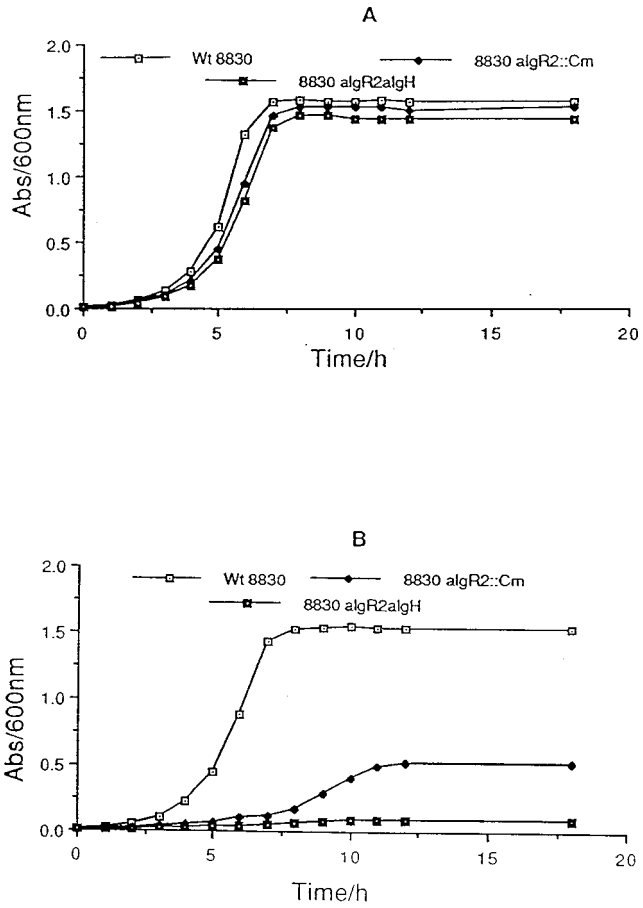

FIG. 4. Effect of 0.1% Tween 20 on growth of the parental strain and the *algR2* and *algR2 algH* mutant strains. The open squares represent 8830, the closed diamonds represent 8830R2::Cm, and the closed squares represent 8830R2::Cm*algH*. The curves in panel A were generated in the absence of Tween 20, while the data in panel B were generated in the presence of 0.1% Tween 20. Abs, absorbance.

of oil (13). When the growth medium of the parental strain, 8830, was assayed for rhamnose-containing biosurfactant, very little could be detected. However, a mutation in *algR2* resulted in a large increase in biosurfactant production (Table 5). By introducing a second mutation, in *algH*, the levels of biosurfactant were increased from that found for the *algR2* mutant. The presence of *algR2* on a plasmid in either the *algR2* or the *algR2 algH* mutant reduced the biosurfactant levels almost to parental levels, implying a repressing activity similar to that of the proteases.

DISCUSSION

In this report we have described the identification and cloning of another gene in *P. aeruginosa* in addition to *algR2* which regulates Scs and Ndk. Although nonmucoid at 37°C, an *algR2*::Cm null mutant was still able to produce alginate at 30°C or upon prolonged incubation at 37°C. This was due to the presence of a second gene, *algH*, which could inefficiently substitute for *algR2* in alginate production at 37°C. The exact mechanism by which AlgR2 regulates the levels of Scs-Ndk complex as well as the *algD* promoter is unknown at present. The significance of Scs and Ndk regulation in alginate biosynthesis has been discussed previously (28, 29). It is interesting

TABLE 2. Alginate production by *algR2* and *algR2 algH* mutants of *P. aeruginosa*[a]

Strain	Amt of alginate formed (mg/plate)
8830	12
8830R2::Cm	<0.5
8830R2::Cm*algH*	<0.5
8830R2::Cm*algH*/*algR2*	14.4

[a] The respective strains were grown on *Pseudomonas* isolation agar plates at 37°C for 24 h. Cells and alginate were scraped from the surface by washing the plates three times with 0.9% NaCl, and alginate was quantitated as described by May and Chakrabarty (21).

TABLE 4. Protease production in *algR2* and *algR2 algH* mutants[a]

Strain	Protease activity [U/ml (%)]
8830	2,870 (100)
8830R2::Cm	8,770 (306)
8830R2::Cm*algH*	9,610 (335)
8830R2::Cm/*algR2*	2,370 (83)
8830R2::Cm*algH*/*algR2*	2,050 (71)

[a] *algR2* was supplied in *trans* on pJK662. The percent values are relative to 8830. One unit of activity is defined as 1 μg of tyrosine released (measured by A_{345}) from the substrate per ml of culture supernatant (2, 3). All strains were grown in L broth at 37°C for 24 h.

that the *algR2 algH* double mutant had undetectable levels of Ndk and was nonmucoid at any temperature tested. At present, it is not clear if Ndk is directly involved in alginate synthesis. Our laboratory is currently making a mutant carrying a null mutation in the *ndk* gene of *P. aeruginosa* to determine if it has a nonmucoid phenotype. While the *ndk* gene has been shown to be essential in some organisms, such as *Myxococcus xanthus* (25), we believe that it may be possible to isolate an *ndk* mutant of *P. aeruginosa* because of the presence of the Tween 20-sensitive enzyme(s) which can supply Ndk function.

In addition to the role of AlgR2 and AlgH in regulating alginate production (Table 2), we have shown that both genes regulate other virulence factors as well. The production of siderophore in *P. putida* WCS358 has been studied, and it was discovered that a protein 58% identical to AlgR2, PfrA, positively regulates siderophore production in response to iron deprivation (37). The role of siderophore under iron-limiting conditions is to bind iron in the environment, which can then be transported into and utilized by the cell. PfrA could complement a *P. aeruginosa* mutant carrying a point mutation in *algR2* (strain 8882) with respect to alginate production, but *algR2* could only weakly complement a *pfrA* mutant with respect to siderophore production. Furthermore, it was shown that strain 8882 was deficient in production of its siderophore, producing only 39% of the parental level (37). Iron is required for growth by all living organisms; sequestering iron from invading bacteria is an important host defense. Here, we were able to demonstrate that the *algR2* insertion mutant 8830R2::Cm produced lower levels of siderophore as expected, but the level was further reduced in the *algR2 algH* double mutant, indicating a role for both AlgR2 and AlgH in siderophore regulation in *P. aeruginosa.*

Another secreted product found to be regulated by *algR2* and *algH* is protease. At least three major extracellular proteases have been determined to be present in *P. aeruginosa*, including alkaline protease and elastase, all of which can act on casein as a substrate (32). While we have not distinguished the exact species of protease being produced in this study, some conclusions concerning the role of proteases in general can be drawn. Proteases are believed to be important virulence factors in bacterial infections, including those involving *P. aeruginosa*. Several functions are served by proteases, including inhibition of host immune responses via cleavage of host immunoglobulins and nonspecific action against polymorphonuclear leukocytes (40). However, it was found that protease is negatively regulated by AlgR2 and AlgH, unlike alginate and siderophore, suggesting that the need for extracellular protease is not as great when the cell is producing alginate and siderophore. At present, we do not know if the decreased activity of siderophore and the increased level of protease in *algR2* mutants or *algR2 algH* double mutants are controlled at the transcriptional level or, perhaps, at the stage of export. Similarly, whether the effect of AlgR2 on *algD* expression or Ndk formation is at the level of transcription or of posttranscriptional stages is also not known. Experiments aimed at further defining this effect are under way in our laboratory. Previous work by Mohr et al. (23) has shown that expression of elastase was inversely related to the mucoid status of the cell; nonmucoid cells showed high levels of *lasB* (encoding elastase) promoter activity, while mucoid cells showed low levels of promoter activity. Using a mutant carrying a null mutation in the *algR1* gene, the authors also showed that a mutation in *algR1*, while rendering the cells nonmucoid, had no effect on *lasB* expression. Furthermore, rhamnolipid surfactant synthesis in *P. aeruginosa* has been shown to be under the control of the *rhlR* gene (26). Interestingly, *rhlR* mutants of *P. aeruginosa* also fail to produce elastase (26). At present, the physiological effects of altered siderophore, protease, or rhamnolipid levels in *algR2* mutants or *algR2 algH* double mutants are unknown. Most of these products are primarily regulated through their own regulators (23, 26, 37); however, *algR2* and *algH* appear to also regulate their extracellular levels, perhaps through a common but indirect mechanism. Our data indicate the multiple controls that *P. aeruginosa* maintains over alginate, siderophore, elastase, and biosurfactant production.

Coordinate regulation of other virulence genes by alginate regulatory genes is not limited to *algR2*. Neuraminidase expression has also been shown to be under the control of *algR1* and *algR2* (4). It will be interesting to determine if other

TABLE 3. Effects of *algR2* and *algR2 algH* mutations on siderophore production[a]

Strain	Siderophore concn [μM (%)]
8830	221 (100)
8830R2::Cm	175 (79)
8830R2::Cm*algH*	106 (46)
8830R2::Cm/*algR2*	210 (95)
8830R2::Cm*algH*/*algR2*	233 (105)

[a] *algR2* was supplied in *trans* on pJK662. The percent values are relative to 8830. All strains were grown in L broth at 37°C for 24 h.

TABLE 5. Rhamnolipid surfactant production in *algR2* and *algR2 algH* mutants[a]

Strain	Amt of rhamnolipid produced (U/ml)
8830	180
8830R2::Cm	720
8830R2::Cm*algH*	1,100
8830R2::Cm/*algR2*	Not detectable
8830R2::Cm*algH*/*algR2*	310

[a] *algR2* was supplied in *trans* on pJK662. One unit is defined as 1 cm^2 of clearing of an oil layer on an agar plate per ml of culture supernatant. All strains were grown in liquid *Pseudomonas* isolation medium at 37°C for 24 h.

virulence factors and enzymes are controlled by other alginate regulatory genes, such as *algR1*, *algB*, and *algU* (*algT*). Regulation of several enzymes (Scs and Ndk) and virulence factors (alginate, siderophore, rhamnolipid, and protease) by *algR2* and *algH* suggests that these genes act as global regulators of several functions.

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