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# Characterization of the Genes Coding for the Putative Sigma Factor AlgU and Its Regulators MucA, MucB, MucC, and MucD in *Azotobacter vinelandii* and Evaluation of Their Roles in Alginate Biosynthesis

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**The study of the biosynthesis of alginate, the exopolysaccharide produced by *Azotobacter vinelandii* and *Pseudomonas aeruginosa*, has biotechnological and medical significance. We report here the identification of the *A. vinelandii* genes coding for the putative sigma factor AlgU and its negative regulators MucA and MucB through the suppression of the highly mucoid phenotype of an *A. vinelandii* strain by a plasmid encoding MucA and MucB. The sequences of the *A. vinelandii algU*, *mucA*, and *mucB* genes are highly homologous to those of the corresponding *P. aeruginosa* genes, AlgU shows 93% identity, and MucA and MucB are 64.4 and 63.9% identical, respectively. Forming part of the same operon as *algU*, *mucA*, and *mucB*, two additional genes (*mucC* and *mucD*) were identified and sequenced; the product of the former gene is homologous to ORF4 of *Photobacterium* sp. strain SS9, and that of the latter gene belongs to the HtrA serine protease family. Interestingly, the nonmucoid *A. vinelandii* UW136 had a 0.9-kb insertion within the *algU* gene. A strong correlation between AlgU activity and alginate production by *A. vinelandii* was also found, as reflected in the level of *algD* transcription.**

*Azotobacter vinelandii* is a gram-negative soil bacterium which in its free-living state is capable of fixing nitrogen under aerobic conditions (22). Under adverse environmental conditions, *A. vinelandii* undergoes a differentiation process leading to the formation of desiccation-resistant cysts. The dormant cells within cysts are able to survive in dry soil for long periods. The encystment process takes approximately 5 days, and the mature cysts are surrounded by two capsule-like layers containing a high proportion of the exopolysaccharide alginate (40). The production of this polysaccharide has been shown to be involved in the differentiation process, since a mutant impaired in alginate production is unable to form desiccation-resistant cells (3).

Considerable information about alginate biosynthesis and its regulation is available on the basis of the studies in *Pseudomonas aeruginosa*. The interest in this bacterium is motivated by the role that alginate plays in the pathogenesis of the lungs of cystic fibrosis patients. Respiratory tract infections with mucoid *P. aeruginosa* strains, which produce copious amounts of alginate, are the major contributing factor causing high morbidity and mortality in cystic fibrosis. The alginate biosynthetic pathways are very similar in *A. vinelandii* (3, 26, 37) and *P. aeruginosa* (4, 33).

In *P. aeruginosa*, the *algD* gene, which codes for the rate-limiting enzyme GDP-mannose dehydrogenase, is located in a biosynthetic cluster which contains the genes coding for the enzymes involved in alginate synthesis, with the exception of *algC*, which codes for the second enzyme in this biosynthetic route (4, 33). This gene cluster appears to be transcribed as one operon (6). At the beginning of the cluster is *algD*, which is controlled by AlgR (9, 10) and possibly by AlgB (17, 18, 41) proteins. The recently identified alternative sigma factor AlgU (11, 12, 19, 38), which is also known as AlgT (13), is responsible for the initiation of *algD* and *algR* and its own transcription (30, 39). This sigma-like factor is similar to the *Escherichia coli* and *Salmonella typhimurium* $\sigma^E$ proteins (14, 19, 32). AlgU and $\sigma^E$ are interchangeable in *P. aeruginosa* (42). They recognize similar promoter canonical sequences, and they are involved in the expression of genes determining resistance to heat and reactive oxygen species (12, 15, 20, 32). AlgU activity is negatively regulated by the products of the *mucA* and *mucB* genes; this regulation is at the posttranscriptional level, with these proteins possibly acting as anti-sigma factors (12). The mucoidy of several *P. aeruginosa* isolates from cystic fibrosis patients is caused by a mutation in the *mucA* gene (31). In addition, insertional inactivation of the *mucB* gene has been found to result in a mucoid phenotype (30).

In *Photobacterium* sp. strain SS9, a gene cluster carrying homologs of *algU*, *mucA*, *mucB*, and *mucC* has been recently described (2, 5). The fourth gene in this cluster, designated ORF4, has been reported to code for a protein which seems to participate in the control of adapted growth at cold temperature and high pressure (5).

We have previously reported the cloning and characterization of several *A. vinelandii* genes involved in alginate biosynthesis (3, 26). Important structural and potential regulatory similarities with the corresponding *P. aeruginosa* genes have been observed. Closely linked to *algD*, other genes involved in alginate metabolism have been identified (26): *algL*, which codes for alginate lyase, and *algA*, which codes for the bifunctional enzyme phosphomannose isomerase-guanosine diphospho-D-mannose pyrophosphorylase. Analyses of these genes

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TABLE 1. Bacterial strains and plasmids used in this work

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *A. vinelandii* | | |
| ATCC 9046 | Alg$^+$ *muc-1* | Collection |
| WI12 | ATCC 9046; *algD-lacZ* | 3 |
| RSD1 | ATCC 9046; *algD* mutant | 3 |
| UW136 | UW; Rif$^r$ | 21 |
| U5 | UW136; *algD-lacZ* | 3 |
| *P. aeruginosa* | | |
| PAO568 | *mucA2 leu-38* Alg$^+$ | 16, 31 |
| PAO578 | *mucA22* Alg$^+$ | 16, 31 |
| PAO579 | *muc-23* Alg$^+$ | 16 |
| Plasmids | | |
| pVDZ′2 | IncP1; *mob*$^+$ *tra lacZ′* (*lacZa*) Tc$^r$ | 8 |
| pDMU4/76 | pVDZ′2; *P. aeruginosa algU*$^+$ *mucA*$^+$ *mucB*$^+$ | 29 |
| pDMUM13 | Deletion derivative of plasmid pDMU4/76; *algU*$^+$ | 29 |
| pCP13 | RK2; *mob*$^+$ *tra* Tc$^r$ Km$^r$ | 7 |
| pSMU865 | pCP13; *A. vinelandii algU mucA*$^+$ *mucB*$^+$ *mucC*$^+$ *mucD*$^+$ | This work |
| pSMU588 | pCP13; unidentified UW136 genes which suppress ATCC 9046 mucoidy | This work |
| pSMU986 | pCP13; unidentified UW136 genes which suppress ATCC 9046 mucoidy | This work |

have indicated that *algL* and *algA* form part of an operon transcribed independently of the *algD* promoter (26). However, despite these observations indicating similarities in the structural genes, regulation of alginate production in *A. vinelandii* has remained largely unexplored.

In this study, we present the isolation of several loci affecting alginate expression in *A. vinelandii* and the cloning and characterization of the *A. vinelandii* genes corresponding to the *algU*, *mucA*, and *mucB* genes of *P. aeruginosa*. These genes seem to play a role in alginate production in *A. vinelandii* similar to that in *P. aeruginosa*. We also present evidence suggesting that AlgU is involved in the expression of *A. vinelandii* resistance to superoxide-generating compounds. The sequences of two new genes (*mucC* and *mucD*), which form part of the putative regulatory cluster headed by *algU*, are also reported. The predicted gene product of *mucC* is homologous to that of *P. aeruginosa mucC* (2) and ORF4 of *Photobacterium* strain SS9 (5), and *mucD* is part of the HtrA family of serine proteases (25), which also includes *P. aeruginosa mucD* (2).

## MATERIALS AND METHODS

**Microbiological procedures.** The bacterial strains and plasmids used in this work are listed in Table 1. *A. vinelandii* strains were routinely grown on BS medium (21) at 30°C. *P. aeruginosa* strains were grown on LB medium (34) or on PIA (Difco) at 37°C. The antibiotic concentrations (in micrograms per milliliter) used for *A. vinelandii* and *P. aeruginosa*, respectively, were 30 and 30 (chloramphenicol), 10 and not used (kanamycin), not used and 50 (neomycin), and 10 and 150 (tetracycline).

Triparental or biparental *A. vinelandii* matings were done as reported previously (3). *A. vinelandii* transformation was done as reported by Bali et al. in 1992 (1).

Alginate production was measured by a method described previously (24). Sensitivity to killing by paraquat was determined by measuring the diameter of the zone of killing surrounding 6-mm-diameter disks impregnated with 10 μl of 1.9% paraquat solution. The disks were placed on 2 ml of soft agar layer containing 0.1 ml of an *A. vinelandii*-saturated culture; these cultures contained equal amount of cells as determined by protein concentrations. β-Galactosidase activity was determined as reported by Miller (34) (1 unit corresponding to 1 nM *o*-nitrophenyl-β-D-galactoside hydrolyzed per min per mg of protein). All measurements were done in triplicate.

**Total DNA library construction.** Total genomic DNA from *A. vinelandii* UW136 was partially digested with *Sau*3A and was ligated to cosmid pCP13 (7) digested with *Bam*HI endonuclease. Cosmids were packaged onto λ heads and transfected as described previously (27). Approximately 5,000 clones were obtained, which represent at least 10 times the size of the bacterial genome.

**Nucleic acid procedures.** DNA isolation and cloning, Southern blotting, and nick translation procedures were carried out as described previously (27). The *P. aeruginosa algU*, *mucA*, and *mucB* gene fragment used as a probe for hybridization with plasmid pSMU865 was obtained from plasmid pDMU4/76, which was described previously (28). The plasmid pSMU865 region homologous to the *P. aeruginosa algU*, *mucA*, and *mucB* genes was subcloned on plasmid pSKII (Stratagene), and its nucleotide sequence was determined with a Sequenase sequencing kit (U.S. Biochemicals). On the basis of this sequence, a series of oligonucleotides were devised and used for direct sequencing of PCR products with an AmpliTaq cycle sequencing kit (Perkin-Elmer) and $^{32}$P-labeled primers. To obtain the sequence of the 5′ end of the *algU* gene, an oligonucleotide based on the nucleotide sequence of the *P. aeruginosa nad-2* gene which is immediately upstream of *algU* gene was designed; the sequence between this gene and the *algU* reading frame was amplified and determined as described above.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the *A. vinelandii* UW136 *algU*, *mucA*, *mucB*, *mucC*, and *mucD* genes reported here have been deposited in the GenBank database under accession number bankit 6399 U30799. For the ATCC 9046 sequences, the accession numbers are U22661 and U22895.

## RESULTS

**Isolation of *A. vinelandii algU*, *mucA*, and *mucB* genes by their effects on alginate biosynthesis.** To isolate some of the *A. vinelandii* genes which exerted a negative control on alginate production, possibly including those corresponding to *mucA* and *mucB* (29–31), we selected cosmid clones from a genomic library made from the nonmucoid strain UW136 which suppressed alginate production of a highly mucoid strain (ATCC 9046). This strategy for the isolation of genes involved in alginate regulation was based on the following premises. (i) *A. vinelandii* ATCC 9046 is highly mucoid, and this increased alginate production is reflected in an increase in the level of *algD* transcription compared with those of other *A. vinelandii* strains (3). (ii) On the basis of the regulation of *P. aeruginosa* alginate production, in which mutations on the *mucA* or *mucB* locus cause conversion to a mucoid phenotype, it seemed possible that a similar regulatory mechanism exists in *A. vinelandii.*

By this approach, three cosmids (pSMU588, pSMU865, and pSMU986) were isolated from a genomic library of the nonmucoid strain UW136, which reduced the mucoidy of strain ATCC 9046. The amounts of alginate produced by the ATCC 9046 derivatives carrying these cosmids are indicated in Table 2.

To further ascertain that the suppression of mucoidy in *A. vinelandii* by the isolated cosmids might resemble the effect and principles previously observed for *P. aeruginosa*, we also

TABLE 2. Effects of *P. aeruginosa algU*$^+$, *mucA*$^+$, and *mucB*$^+$ and *A. vinelandii algU*, *mucA*$^+$, and *mucB*$^+$ plasmids on alginate production

| Plasmid | Genotype[a] | Alginate production (μg/mg)[b] | |
|---|---|---|---|
| | | ATCC 9046 (*algU mucA mucB muc-1*) | UW136 (*algU-mucA mucB*) |
| None | | 151.0 ± 7.5 | 4.0 ± 0.6 |
| pVDZ′2 | Vector | 144.1 ± 9.5 | 5.1 ± 0.1 |
| pDMU4/76 | *algU mucA mucB*$_P$ | 8.8 ± 0.1 | 4.1 ± 0.6 |
| pCP13 | Vector | 159.0 ± 9.6 | 3.8 ± 0.3 |
| pSMU865 | *algU-mucA mucB*$_A$ | 44.4 ± 3.2 | 4.2 ± 0.6 |
| pSMU588 | Unidentified | 52.9 ± 4.2 | ND |
| pSMU986 | Unidentified | 57.1 ± 3.2 | ND |
| pDMUM13 | *algU*$_P$ | ND | 18.6 ± 2.0 |

[a] The subscripts A and P denote that the genes are from *A. vinelandii* and *P. aeruginosa*, respectively.

[b] Alginate production expressed in micrograms of alginate per milligram of cells (wet weight) ± standard error of the mean. ND, not done.

determined whether mucoidy was suppressed by the transfer of a plasmid expressing the wild-type *P. aeruginosa mucA* and *mucB* genes (pDMU4/76) in ATCC 9046. As shown in Table 2, alginate production by strain ATCC 9046 was almost completely abrogated by plasmid pDMU4/76, in keeping with the previously reported observations for *P. aeruginosa* (29).

**Sequence analysis of *A. vinelandii algU*, *mucA*, *mucB*, *mucC*, and *mucD* genes.** Since the different cosmids displayed suppressing activities on ATCC 9046 mucoidy, we reasoned that if one of the clones carried the *algU* gene and its downstream regulators, it might hybridize with the *P. aeruginosa* probes. By Southern blot analysis, it was shown that plasmid pSMU865 contained a 10-kb *Eco*RI fragment which hybridized to the *P. aeruginosa algU*, *mucA*, and *mucB* genes present in plasmid pDMU4/76 (data not shown). On the basis of this homology, the 10-kb *Eco*RI fragment was subcloned and used as a template to sequence the putative *A. vinelandii algU* and downstream genes.

This hypothesis was borne out. The nucleotide sequence of the region corresponding to the *algU*, *mucA*, and *mucB* genes in the nonmucoid *A. vinelandii* strain UW136 is shown in Fig. 1. Comparison of the deduced amino acid sequence with the homologous protein sequences from *P. aeruginosa* and *E. coli* is shown in Fig. 2. Surprisingly, no complete open reading frame encoding *algU* was found in the nonmucoid strain UW136. MucA and MucB displayed 64.4 and 63.9% identity, respectively, with the corresponding products in *P. aeruginosa* (Fig. 2B and C).

Two additional genes downstream of *mucB* and forming part of the same operon were identified (Fig. 1). The gene following *mucB* was termed *mucC*. The predicted protein product of *mucC* is homologous to the corresponding polypeptide encoded by *Photobacterium* strain SS9 ORF4 (Fig. 2D). The most distant gene in this operon was designated *mucD*, and it showed high similarity to the family of HtrA-like serine proteases (Fig. 2E).

**Identification of an *algU* mutation in the nonmucoid *A. vinelandii* strain UW136.** Although no apparent complete *algU* gene was present in UW136, some similarity to *algU* in *P. aeruginosa* was observed; this sequence similarity stopped between nucleotides 160 and 161 in *A. vinelandii* UW136 DNA. To determine whether lack of the complete *algU* gene in UW136 was typical of *A. vinelandii* or was characteristic of this particular nonmucoid strain, the nucleotide sequence of the corresponding region in the mucoid *A. vinelandii* strain ATCC 9046 was determined. When this sequence was analyzed, a complete *algU* gene was observed (Fig. 1). Comparison of the sequences from ATCC 9046 and UW136 showed that the *algU* gene from strain UW136 had a putative insertion sequence of approximately 0.9 kb between the codons encoding amino acids 55 and 56 of AlgU. Figure 3 shows the sequences of the inverted repeats corresponding to the ends of the putative insertion sequence in the *algU* gene of UW136. These inverted repeats were flanked by an 8-bp direct repeat typical of transposition events.

The *mucA* and *mucB* DNA sequences of the highly mucoid *A. vinelandii* strain ATCC 9046 were determined, and no changes could be detected with respect to MucA or MucB protein encoded by the *algU* mutant UW136. Additionally, analysis of the DNA sequence upstream of the *algU* initiation codon suggested the presence of putative transcription regulatory sequences (data not shown). Two sequences were identified which displayed similarity between the −10 and −35 regions of the promoters recognized by the RNA polymerase holoenzyme containing the alternative sigma factor $\sigma^E$ (12, 14).

**Evaluation of the roles of AlgU, MucA, and MucB in alginate production.** To determine whether the nonmucoid phenotype of *A. vinelandii* UW136 was due to inactivation of the *algU* gene by the putative insertion sequence found in this strain, an active *P. aeruginosa algU* gene (plasmid pDMUM13) was transferred into *A. vinelandii* UW136. It was found that the *P. aeruginosa algU* gene enabled strain UW136 to produce alginate (Table 2). This confirmed the possibility that *algU* plays a positive regulatory role in alginate production in *A. vinelandii.*

Plasmid pSMU865 reduced alginate production in strain ATCC 9046 (Table 2), presumably by increasing the concentrations of MucA and MucB relative to that of AlgU. To further explore this possibility, plasmid pSMU865 was transferred to several well-characterized *P. aeruginosa* strains carrying mapped *muc* mutations. PAO568 (*mucA2*) and PAO578 (*mucA22*) can be suppressed by the expression in *trans* of *P. aeruginosa mucA* and *mucB* genes (29–31). Table 3 shows that alginate production by *P. aeruginosa* PAO568 and PAO578 is significantly reduced in the presence of plasmid pSMU865. The suppression of mucoidy by plasmid pSMU865 is also apparent with strain PAO579, which carries a mutation in a locus that has not yet been identified, and is also suppressed by the expression of *P. aeruginosa* genes *mucA* and *mucB* (Table 3).

**Role of AlgU in the transcription of the *A. vinelandii algD* gene.** It was reported that the level of *algD* transcription, which was measured as β-galactosidase activities of the derivatives carrying an *algD-lacZ* gene fusion of the highly mucoid ATCC 9046 and nonmucoid UW136 strains (WI12 and U5, respectively), is related to their alginate production (3). Our results presented here, together with the presence in strain ATCC 9046 of an *algD* mRNA start site which mapped in the correct position downstream of the $\sigma^E$ canonical sequence and upstream of the *algD* gene and the absence of this mRNA in the *algU* strain UW136 (3), suggest that AlgU is the sigma factor involved in the initiation of *algD* transcription in *A. vinelandii*. To obtain further evidence in support of this hypothesis, we determined the level of *algD* transcription in the *algU* mutant UW136 and in the *algU*$^+$ strain ATCC 9046. To measure *algD* transcription, we used strains WI12 and U5, which carry an *algD-lacZ* gene fusion and are derived from strains ATCC 9046 and UW136, respectively. The level of *algD* transcription correlates with the level of alginate production by these strains (Table 4). As shown in Table 4, the level of *algD* expression

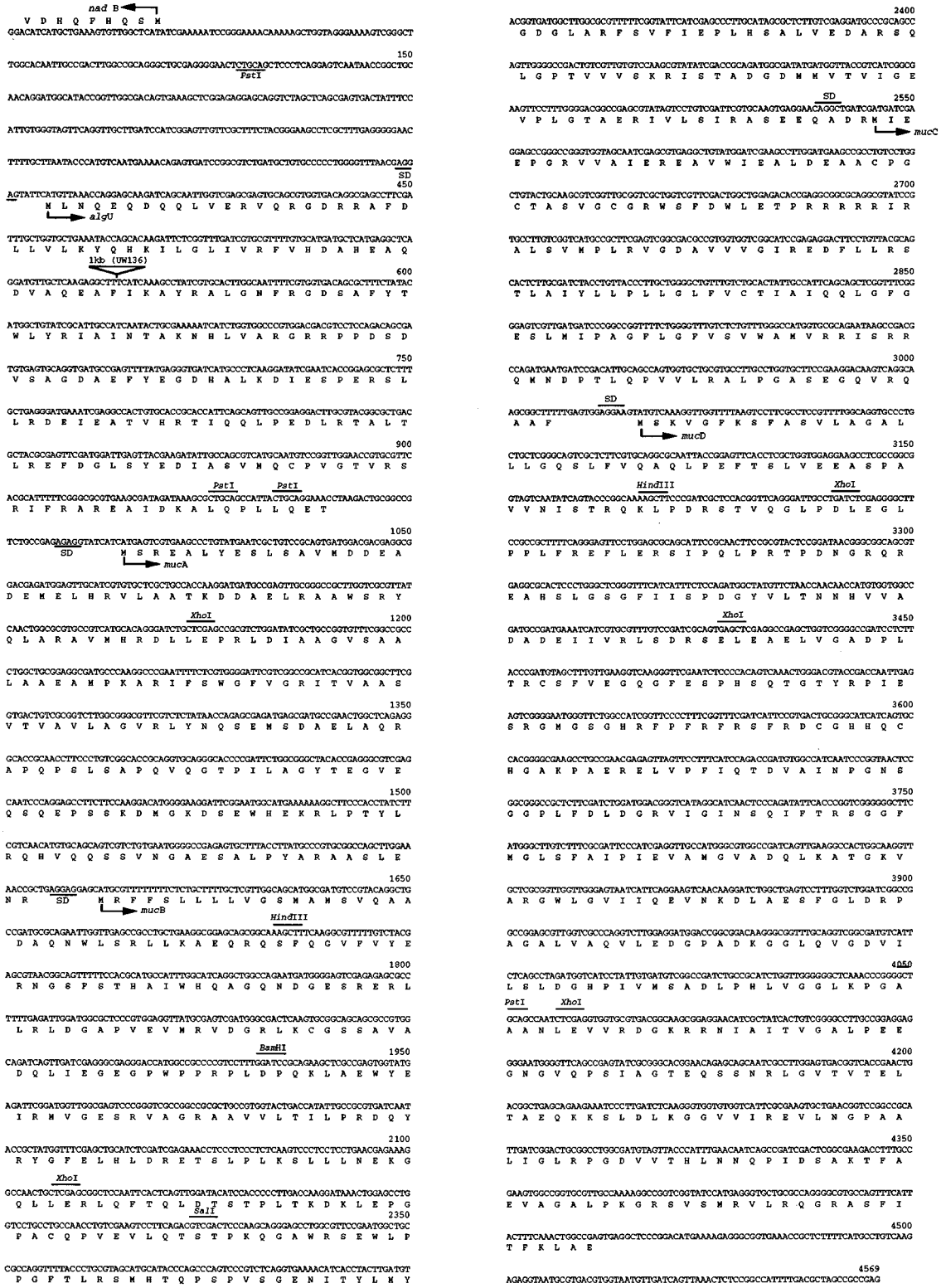
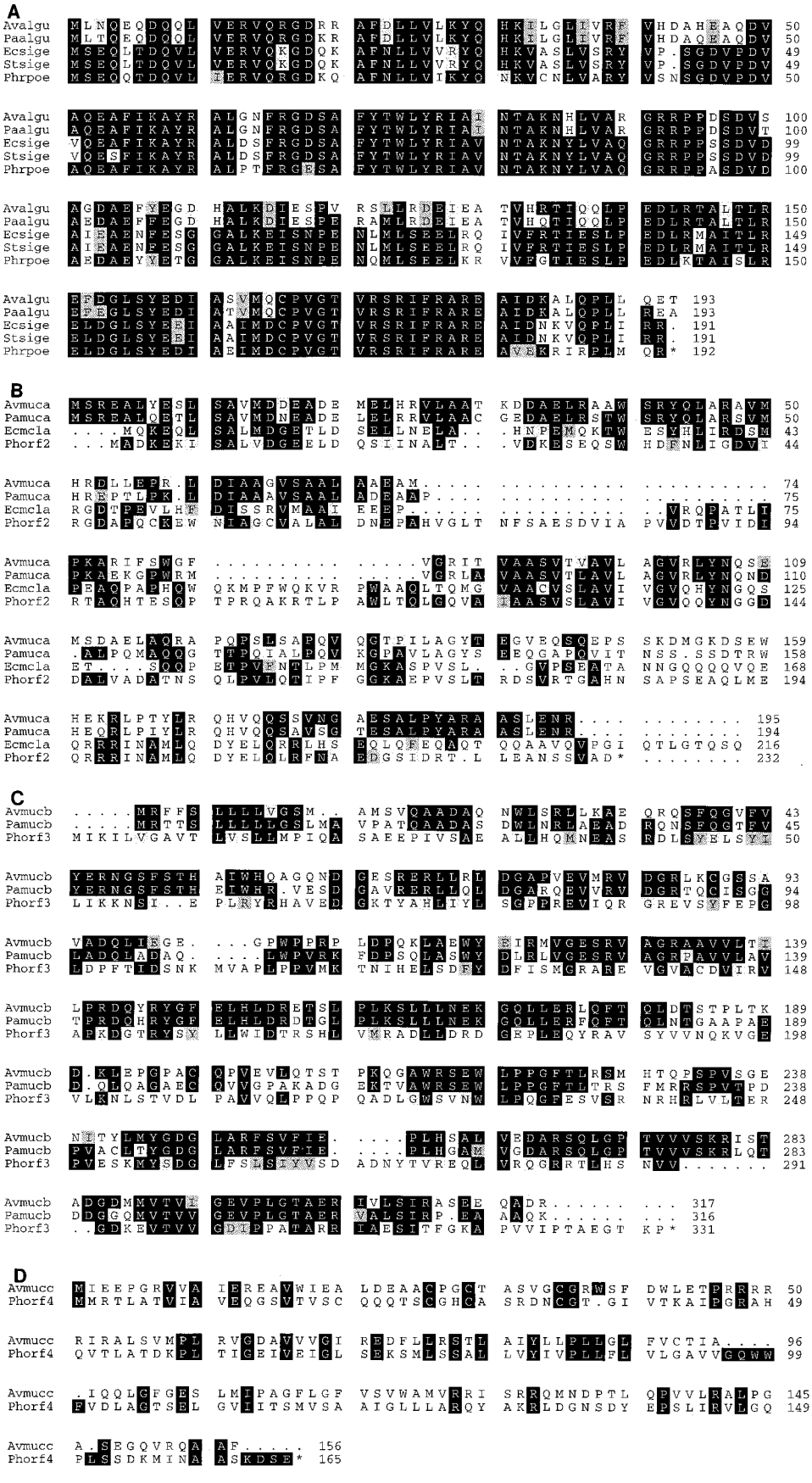



FIG. 1. Nucleotide sequences of *A. vinelandii* UW136 *algU*, *mucA*, *mucB*, *mucC*, and *mucD* genes and their predicted amino acid coding sequences. The nucleotide sequence from 1 to 2547 (end of *mucB*) of the *algU*$^+$ strain ATCC 9046 was determined and is identical to the UW136 DNA sequence, except for a T-to-A change in position 1524 (which is conservative). The complete *algU mucABCD* sequence for UW136 was determined, and the position of the 1-kb insertion sequence is shown.



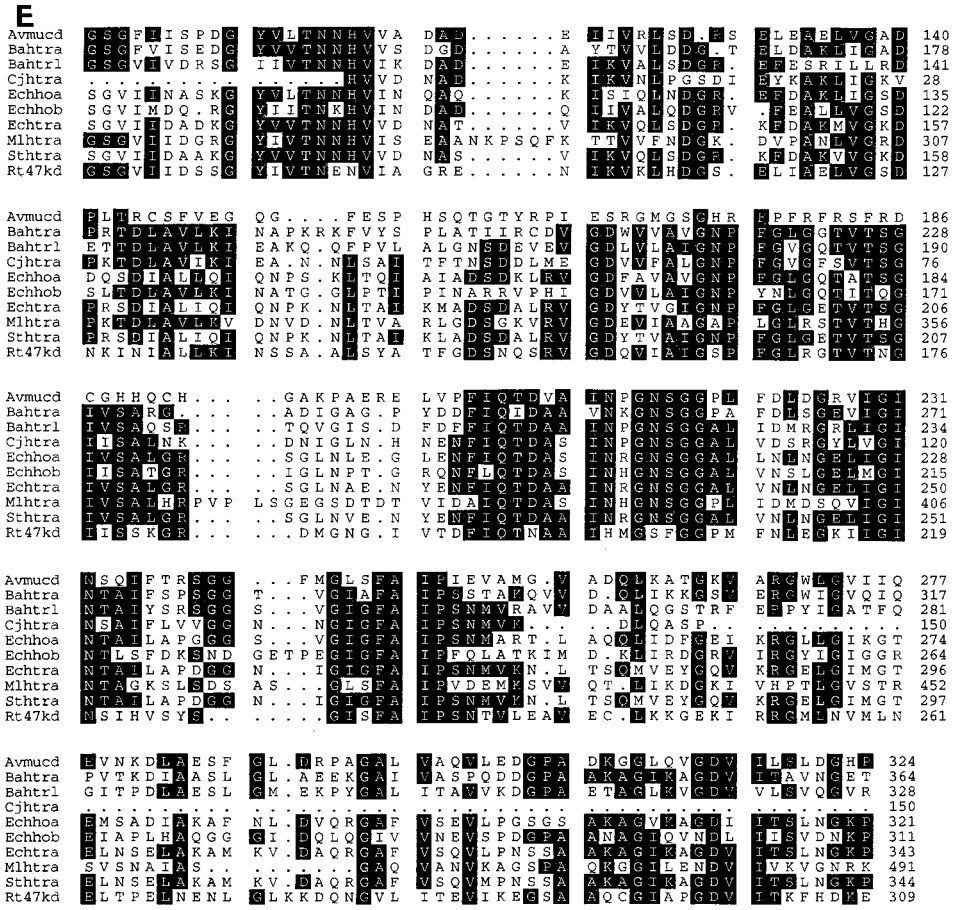



FIG. 2. Alignments of the predicted amino acid sequences of AlgU, MucA, MucB, MucC, and MucD from *A. vinelandii* and their reported homologs. (A) Alignment of the *A. vinelandii* AlgU protein (Avalgu) with the homolog protein of *P. aeruginosa* (Paalgu), *E. coli* (Ecsige), *Salmonella typhimurium* (Stsige), and *Photobacterium* strain SS9 (Phrpoe); (B) alignment of the *A. vinelandii* MucA protein (Avmuca) with the homolog proteins from *P. aeruginosa* (Pamuca), *E. coli* (Ecmc1a), and *Photobacterium* strain SS9 (Phorf2); (C) alignment of the *A. vinelandii* MucB protein (Avmucb) with the homolog proteins of *P. aeruginosa* (Pamucb) and *Photobacterium* strain SS9 (Phorf3); (D) alignment of the *A. vinelandii* MucC protein (Avmucc) with ORF4 of *Photobacterium* strain SS9 (Phorf4); (E) alignment of the *A. vinelandii* MucD protein (Avmucd) with the Htr family of proteases, which includes *Brucella abortus* HtrA-like proteins (Bahtra and Bahtr1); *Campylobacter jejuni* HtrA (Cjhtra); *E. coli* HhoA (Echhoa), Hhob (Echhob), and HtrA (Echtra); *Mycobacterium leprae* HtrA (Mlhtra); *S. typhimurium* HtrA (Sthtra); and a 47-kDa *Rickettsia tsutsugamushi* protein (Rt47kd). The two shades of gray represent increasing levels of conservation in cases of nonidentical amino acids. The alignment was done by using the PILEUP program, University of Wisconsin Genetics Computer Group.

was much higher in WI12 (*algU*$^+$) than in U5 (*algU* mutant). When *P. aeruginosa algU* was introduced into U5, the level of *algD* transcription was slightly increased. Plasmid-borne *algU* showed no increase in the level of transcription of the *algD* gene in the *algU*$^+$ genetic background (strain WI12). The results presented suggest that AlgU activity is a contributing factor in *algD* transcription in *A. vinelandii.*

**Role of AlgU in resistance to reactive oxygen species in *A. vinelandii.*** To determine whether *A. vinelandii* AlgU was involved in tolerance to reactive oxygen species, we determined

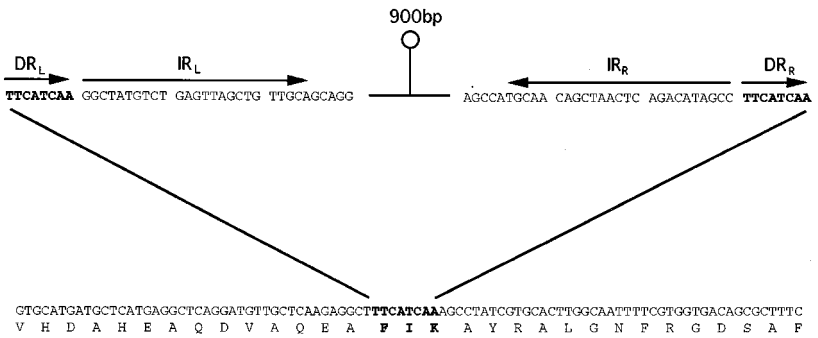



FIG. 3. Inverted-repeat (IR) sequences of the putative insertion element inserted in the *algU* gene of *A. vinelandii* UW136 and the flanking 8-bp direct repeats (DR).

TABLE 3. Effects of plasmid pSMU865 carrying *A. vinelandii algU*, *mucA*$^+$, and *mucB*$^+$ genes and plasmid pDMU4/76 carrying the corresponding *P. aeruginosa* genes on alginate production by mucoid *P. aeruginosa* strains

| Plasmid[a] | Alginate production (μg/mg)[b] | | |
|---|---|---|---|
| | PAO568 (*mucA2*) | PAO578 (*mucA22*) | PAO579 (*muc-23*) |
| pCP13 | 105.0 ± 7.5 | 143.2 ± 9.1 | 138.0 ± 6.8 |
| pSMU865 | 54.2 ± 3.8 | 40.0 ± 6.0 | 9.3 ± 1.7 |
| pDMU4/76 | >0.8 | >0.8 | >0.8 |

[a] The relevant genotypes of the plasmids are shown in Table 2.
[b] Alginate production expressed in micrograms of alginate per milligram of cells (wet weight) ± standard error of the mean. The below limit of detection of this method is 0.8 μg/mg of cells (wet weight).

sensitivity to paraquat, a compound known to generate superoxide intracellularly. Strains UW136 (*algU* mutant) and ATCC 9046 (*algU*$^+$) containing plasmids pDMUM13 (*P. aeruginosa algU*$^+$) and pSMU865 (*A. vinelandii algU mucABCD*$^+$), respectively, were used. The level of resistance of strain UW136 to paraquat was increased by the expression of *P. aeruginosa algU*. The level of resistance of strain ATCC 9046 was reduced by the expression of *A. vinelandii mucABCD* genes (Table 5); this reduction is not due to the reduced permeability to paraquat by the presence of alginate, since the *algD* mutant derived from strain ATCC 9046 (RSD1), which is completely impaired in alginate production (3), has the same sensitivity to paraquat as strain ATCC 9046 (data not shown). However, the zones of inhibition were surprisingly larger in the *algU*$^+$ strain ATCC 9046 than in UW136, suggesting that these strains have other genotypic and phenotypic differences.

## DISCUSSION

The strategy that we followed in this study for the isolation of *A. vinelandii mucA* and *mucB* genes, i.e., suppression of the highly mucoid phenotype of strain ATCC 9046 by genes from the nonmucoid strain UW136, suggests that the role of these genes in alginate biosynthesis is possibly similar to that reported for the corresponding *P. aeruginosa* genes. The observation that alginate production is diminished in *A. vinelandii* ATCC 9046 by a plasmid carrying *mucA* and *mucB* genes from *P. aeruginosa* (plasmid pDMU4/76) or from *A. vinelandii* (plasmid pSMU865) suggested that this highly mucoid strain might have a mutation in the corresponding genes. However, the DNA sequences of these genes are almost identical in the two *A. vinelandii* strains studied, except for a single nucleotide change which is conservative (Fig. 1). It has been shown that in *P. aeruginosa* there are mucoid mutants which do not have lesions in *mucA* and *mucB* genes but are nevertheless suppressed in *trans* by *mucA* and *mucB* genes (16, 29). This appears to be the case for the strain PAO579 studied here, which is also suppressed by *A. vinelandii muc* genes present in plasmid pSMU865. The highly mucoid *A. vinelandii* strain ATCC 9046 closely resembles strain PAO579 in this respect, and this situation underscores the idea that in *A. vinelandii*, as in *P. aeruginosa*, the regulation of alginate production represents a very complex interaction of multiple factors.

We have previously reported that the nonmucoid *A. vinelandii* strain UW136 displays a very low level of expression of the *algD* promoter under all conditions tested. We have proposed that this might be due to a deficiency in one of the regulators involved in *algD* transcription (3). In the present paper, we show that this regulator is the alternative sigma factor AlgU. Strain UW136 contains an insertion sequence interrupting the *algU* gene. The complementation to nonmucoidy of strain ATCC 9046 with plasmid pSMU865 also suggests that this insertion is not polar on *mucA*, *mucB*, *mucC*, and *mucD* genes encoded in the same operon.

Sequence analysis of the 10-kb *Eco*RI fragment of pSMU865, which showed homology to *P. aeruginosa algU*, *mucA*, and *mucB* genes, permitted the identification in *A. vinelandii* of two additional genes forming part of the same operon (Fig. 1). The homology of the predicted gene product of *mucC* to that of *P. aeruginosa mucC* (2) and ORF4 of *Photobacterium* strain SS9 (5) and the fact that *mucD* is part of the HtrA family of serine proteases (25), which also includes *P. aeruginosa mucD* (2), suggest that they are involved in the response of cells to stress. ORF4 of *Photobacterium* strain SS9 participates in bacterium-adapted growth at low temperatures and under high pressure (5). Mutations in *P. aeruginosa mucD* produce a mucoid phenotype (2). The *E. coli htrA* gene encodes a periplasmic endopeptidase that is essential at temperatures above 42°C (25) and is thus important in cell resistance to heat killing. It has been reported that RNA polymerase containing $\sigma^E$ is the only form of this enzyme that transcribes the *htrA* gene (14). The precise role of MucC and MucD in *A. vinelandii* remains to be investigated.

ORF4 in *Photobacterium* strain SS9 forms part of an operon (5) which is very similar to that described here for *A. vinelandii algU*, *mucA*, *mucB*, *mucC*, and *mucD*. The first gene of the *Photobacterium* sp. operon codes for a putative sigma factor homologous to AlgU, and two genes then follow which are homologous to *mucA* and *mucB*. The fourth gene is ORF4. The location of this gene forming part of the same operon as *rpoE* suggests that it may be involved in the regulation of genes transcribed by RNA polymerase containing the AlgU sigma factor. The fifth gene of the *Photobacterium* strain SS9 operon (ORF5) has no reported homology with *E. coli htrA*.

The genes encoded by plasmids pSMU588 and pSMU986 are not homologous to *algU*, *mucA*, or *mucB* and have not yet been characterized. These cosmids have definite effects on alginate production by *A. vinelandii* ATCC 9046. The *muc-1* mutation of strain ATCC 9046 might affect a gene encoded by one of these plasmids or either *mucC* or *mucD*.

It has been previously reported that the *algD* gene from strain ATCC 9046 is transcribed from two promoters: the above-described promoter transcribed by the RNA polymerase containing the AlgU sigma factor and a promoter recognized by the RNA polymerase containing the $\sigma^D$ subunit (3). The $\sigma^D$ promoter seems to be absent in strain UW136 (3). An alternative explanation for the highly mucoid phenotype of strain

TABLE 4. Alginate production and β-galactosidase activity

| Strain | Genotype[a] | Alginate production (μg/mg)[b] | β-Galactosidase activity[c] |
|---|---|---|---|
| U5 | *algU algD-lacZ*$_A$ | 1.6 ± 0.9 | 63 ± 8 |
| U5/pDMUM13 | *algU*$_A$/*algU*$^+_P$ | 19.8 ± 6.5 | 96 ± 26 |
| WI12 | *muc-1 algD-lacZ*$_A$ | 84.9 ± 15.7 | 1,172 ± 233 |
| WI12/pDMUM13 | *muc-1*$_A$/*algU*$^+_P$ | 44.5 ± 10.4 | 882 ± 141 |

[a] The subscripts A and P denote that the genes are from *A. vinelandii* and *P. aeruginosa*, respectively. Genes carried on a plasmid follow shills.
[b] Alginate production expressed in micrograms of alginate per milligram of cells (wet weight) ± standard error of the mean.
[c] β-Galactosidase activities were determined as reported by Miller (33). One unit corresponds to 1 nM *o*-nitrophenyl-β-D-galactoside hydrolyzed per min per mg of protein (± standard error of the mean).

TABLE 5. Differential sensitivity to killing by paraquat in *A. vinelandii*

| Strain | Genotype[a] | Growth inhibition zone (mm ± SE)[b] |
|---|---|---|
| UW136 | *algU mucA*$^+$ *mucB*$_A$$^+$ | 15.6 ± 0.4 |
| UW136/pDMUM13 | Same as UW136$_A$/*algU*$_P$ | 11.0 ± 0 |
| ATCC 9046 | *algU*$^+$ *mucA*$^+$ *mucB*$^+$ *muc-1*$_A$ | 26.0 ± 0 |
| ATCC 9046/pSMU865 | Same as ATCC 9046/*algU-mucA mucB*$_A$ | 37.0 ± 0.5 |

[a] The subscripts A and P denote that the genes are from *A. vinelandii* and *P. aeruginosa*, respectively. Genes carried on a plasmid follow shills.

[b] Growth inhibition zones are expressed as means of the diameters of inhibition of growth.

ATCC 9046 is that the presence of the $\sigma^D$ *algD* promoter accounts for the higher level of transcription of this gene.

The DNA sequence upstream of *A. vinelandii algU* was obtained by using an oligonucleotide derived from the sequence of the *P. aeruginosa nad-2* gene. This result shows that the DNA sequence homology between these bacteria is not restricted to *alg* genes and that the arrangement of genes in this chromosomal region is also conserved. Interestingly, the *nadB* gene is upstream of *rpoE* in the *E. coli* chromosome (28).

The *A. vinelandii* DNA sequence upstream of the *algU* coding region shows two DNA motifs with the characteristics of the −10 and −35 regions of the promoters transcribed by the RNA polymerase containing $\sigma^E$. This result suggests that the transcriptional regulation of the *algU* gene in *A. vinelandii* is similar to that reported for *P. aeruginosa*, in which AlgU has been found to control its own transcription (32, 39); however, the functionality of the sequences remains to be determined.

We report here that there is a correlation between AlgU activity and alginate production by *A. vinelandii* and that this positive correlation is reflected in the levels of *algD* transcription. In contrast, another *alg* promoter directing the transcription of *algL* and *algA* genes is not dependent on AlgU for its transcription, since we detected alginate lyase activities in the genetic backgrounds of the *algU*$^+$ strain ATCC 9046 that were equal to those in the *algU* mutant UW136 (26). These results are in accordance with the report by Kennedy et al. in 1992 who showed that strain UW (which is the parental strain of strain UW136 studied here) had alginate lyase activity similar to that of the alginate-producing strains (23).

The involvement of the alternative $\sigma^E$ factors in cell resistance to superoxide has been reported for *P. aeruginosa* (32). This effect was evaluated in *A. vinelandii*, and we found a correlation between AlgU activity and resistance to paraquat, which is known to generate superoxide intracellularly. The involvement of AlgU in *A. vinelandii* response to other environmental insults remains to be determined.

One of the aims of our research is the study of the role of alginate biosynthesis in the *A. vinelandii* differentiation process leading to cyst formation; accordingly, we have shown that an *algD* mutant derived from strain ATCC 9046 is completely impaired in cyst formation (3). It has been reported that the nonmucoid *A. vinelandii* strain UW has a defect in cyst formation, since even after prolonged storage it can make only incomplete cyst-like structures (36). We have found that this strain is unable to make desiccation-resistant structures (35). However, it is not clear whether the defect in UW136 affects the differentiation process only via its alginate deficiency or whether it is also caused by its lack of AlgU activity. In this respect, we are currently analyzing at the ultrastructural level the development of cysts in different strains, including strains UW136 and ATCC 9046.

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## REFERENCES

1. **Bali, A., G. Blanco, S. Hill, and C. Kennedy.** 1992. Excretion of ammonium by a *nifL* mutant of *Azotobacter vinelandii* fixing nitrogen. Appl. Environ. Microbiol. **58:**1711–1718.
2. **Boucher, J. C., J. Martínez-Salazar, M. J. Schurr, M. Mudd, H. Yu, and V. Deretic.** 1996. Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologs of the serine protease HtrA. J. Bacteriol. **178:**511–523.
3. **Campos, M.-E., J. M. Martínez-Salazar, L. Lloret, S. Moreno, C. Núñez, G. Espín, and G. Soberón-Chávez.** 1996. Characterization of the gene coding for GDP-mannose dehydrogenase (*algD*) from *Azotobacter vinelandii*. J. Bacteriol. **178:**1793–1799.
4. **Chakrabarty, A. M.** 1991. Molecular genetics and environmental regulation of alginate biosynthesis. Appl. Phycol. **8:**1–6.
5. **Chi, E., and D. H. Bartlett.** 1995. Characterization of a locus important for high pressure adaptation from the barophilic deep-sea bacterium *Photobacterium* SS9. Mol. Microbiol. **17:**713–726.
6. **Chitnis, C. E., and D. E. Ohman.** 1993. Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. Mol. Microbiol. **8:**583–590.
7. **Darzins, A., and A. M. Chakrabarty.** 1984. Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa*. J. Bacteriol. **159:**9–18.
8. **Deretic, V., S. Chandrasekharappa, J. F. Gill, D. K. Chatterjee, and A. M. Chakrabarty.** 1987. A set of cassettes and improved vectors for genetic and biochemical characterization of *Pseudomonas* genes. Gene **57:**61–72.
9. **Deretic, V., R. Dikshit, W. M. Konyecsni, A. M. Chakrabarty, and T. K. Misra.** 1989. The *algR* gene, which regulates mucoidy in *Pseudomonas aeruginosa*, belongs to a class of environmentally responsive genes. J. Bacteriol. **171:**1278–1283.
10. **Deretic, V., J. R. W. Govan, W. M. Konyecsni, and D. W. Martin.** 1990. Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: mutations in the *muc* loci affect transcription of the *algR* and *algD* genes in response to environmental stimuli. Mol. Microbiol. **42:**189–196.
11. **Deretic, V., D. W. Martin, M. J. Schurr, M. H. Mudd, N. S. Hibler, R. Curcic, and J. C. Boucher.** 1993. Conversion to mucoidy in *Pseudomonas aeruginosa*. Bio/Technology **11:**1133–1136.
12. **Deretic, V., M. J. Schurr, J. C. Boucher, and D. W. Martin.** 1994. Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. J. Bacteriol. **176:**2773–2780.
13. **DeVries, C. A., and D. E. Ohman.** 1994. Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternative sigma factor, and shows evidence of autoregulation J. Bacteriol. **176:**6677–6687.
14. **Erickson, J. W., and C. A. Gross.** 1989. Identification of the $\sigma^E$ subunit of *Escherichia coli* RNA polymerase: a second alternate σ factor involved in high-temperature gene expression. Genes Dev. **3:**1462–1471.
15. **Farr, S., and T. Kogoma.** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **55:**561–568.
16. **Fyfe, J. A. M., and J. R. W. Govan.** 1980. Alginate synthesis in mucoid *Pseudomonas aeruginosa*: a locus involved in control. J. Gen. Microbiol. **119:**443–450.
17. **Goldberg, J. B., and T. Dahnke.** 1992. *Pseudomonas aeruginosa* AlgB, which modulates the expression of alginate, is a member of the NtrC subclass of procaryotic regulators. Mol. Microbiol. **6:**59–66.
18. **Goldberg, J. B., and D. E. Ohman.** 1987. Construction and characterization of *Pseudomonas aeruginosa algB* mutants: role of *algB* in high-level production of alginate. J. Bacteriol. **169:**1593–1602.
19. **Hershberger, C. D., R. W. Ye, M. R. Parsek, Z.-D. Xie, and A. M. Chakrabarty.** 1995. The *algT* (*algU*) gene of *Pseudomonas aeruginosa*, a key regulator involved in alginate biosynthesis, encodes an alternative σ factor ($\sigma^E$). Proc. Natl. Acad. Sci. USA **92:**7941–7945.
20. **Johnson, K., I. Charles, G. Dougan, D. Pickard, P. O'Gaora, G. Costa, T. Ali, I. Miller, and C. Hormaeche.** 1991. The role of stress response protein in *Salmonella typhimurium* virulence. Mol. Microbiol. **5:**401–407.
21. **Kennedy, C., R. Gamal, R. Humphrey, J. Ramos, K. Brigle, and D. Dean.** 1986. The *nifH*, *nifM* and *nifN* genes of *Azotobacter vinelandii*: characteriza-

tion by Tn*5* mutagenesis and isolation from pLAFR1 gene banks. Mol. Gen. Genet. **205:**318–325.
22. **Kennedy, C., and A. Toukdarian.** 1987. Genetics of azotobacters: applications to nitrogen fixation and related aspects of metabolism. Annu. Rev. Microbiol. **41:**227–258.
23. **Kennedy, L., K. McDowell, and I. W. Sutherland.** 1992. Alginases from *Azotobacter* species. J. Gen. Microbiol. **138:**2465–2471.
24. **Knutson, C. A., and A. Jeanes.** 1968. A new modification of the carbazole reaction: application to heteropolysaccharides. Anal. Biochem. **24:**470–481.
25. **Lipinska, B., S. Sharma, and C. Georgopoulos.** 1988. Sequence analysis and regulation of the *htrA* gene of *Escherichia coli*: a $\sigma^{32}$-independent mechanism of heat inducible transcription. Nucleic Acids Res. **16:**10053–10067.
26. **Lloret, L., R. Barrato, M.-E. Campos, S. Moreno, J. M. Martínez-Salazar, G. Espín, R. León, and G. Soberón-Chávez.** Unpublished data.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Martin, D. W., and V. Deretic.** Unpublished results.
29. **Martin, D. W., B. W. Holloway, and V. Deretic.** 1993. Characterization of a locus determining the mucoid status of *Pseudomonas aeruginosa*: AlgU shows sequence similarities with a *Bacillus* sigma factor. J. Bacteriol. **175:** 1153–1164.
30. **Martin, D. W., M. J. Schurr, M. H. Mudd, and V. Deretic.** 1993. Differentiation of *Pseudomonas aeruginosa* into the alginate-producing form: inactivation of *mucB* causes conversion to mucoidy. Mol. Microbiol. **9:**495–506.
31. **Martin, D. W., M. J. Schurr, M. H. Mudd, J. R. W. Govan, B. W. Holloway, and V. Deretic.** 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc. Natl. Acad. Sci. USA **90:** 8377–8381.
32. **Martin, D. W., M. J. Schurr, H. Yu, and V. Deretic.** 1994. Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relation to $\sigma^{E}$ and stress response. J. Bacteriol. **176:**6688–6696.
33. **May, T. B., and A. M. Chakrabarty.** 1994. *Pseudomonas aeruginosa*: genes and enzymes of alginate synthesis. Trends Microbiol. **2:**151–157.
34. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431–435. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Moreno, S., R. Najera, J. Guzmán, G. Soberón-Chávez, and G. Espín.** Unpublished results.
36. **Page, W. J.** 1983. Formation of cyst-like structures by iron-limited *Azotobacter vinelandii* strain UW during prolonged storage. Can. J. Microbiol. **29:** 1110–1118.
37. **Pindar, D. F., and C. Bucke.** 1975. The biosynthesis of alginic acid by *Azotobacter vinelandii*. Biochem. J. **152:**617–622.
38. **Schurr, M. J., D. W. Martin, M. H. Mudd, N. S. Hibier, J. C. Boucher, and V. Deretic.** 1993. The *algD* promoter: regulation of alginate production by *Pseudomonas aeruginosa* in cystic fibrosis. Cell. Mol. Biol. Res. **39:**371–376.
39. **Schurr, M. J., H. Yu, J. C. Boucher, N. S. Hibler, and V. Deretic.** 1995. Multiple promoters and induction by heat shock of the gene encoding the alternative sigma factor AlgU ($\sigma^{E}$) which controls mucoidy in cystic fibrosis isolates of *Pseudomonas aeruginosa*. J. Bacteriol. **177:**5670–5679.
40. **Sutherland, I. W.** 1985. Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharides. Annu. Rev. Microbiol. **39:**243–270.
41. **Wozniak, D. J., and D. E. Ohman.** 1994. Transcriptional analysis of the *Pseudomonas aeruginosa* genes *algR*, *algB*, and *algD* reveals a hierarchy of alginate gene expression which is modulated by *algT*. J. Bacteriol. **176:** 6007–6014.
42. **Yu, H., M. J. Schurr, and V. Deretic.** 1995. Functional equivalence of *Escherichia coli* $\sigma^{E}$ and *Pseudomonas aeruginosa* AlgU: *E. coli rpoE* restores mucoidy and reduces sensitivity to reactive oxygen intermediates in *algU* mutants of *P. aeruginosa*. J. Bacteriol. **177:**3259–3268.