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# Role of *Azotobacter vinelandii mucA* and *mucC* Gene Products in Alginate Production

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***Azotobacter vinelandii*** **produces the exopolysaccharide alginate, which is essential for its differentiation to desiccation-resistant cysts. In different bacterial species, the alternative sigma factor $\sigma^E$ regulates the expression of functions related to the extracytoplasmic compartments. In *A. vinelandii* and *Pseudomonas aeruginosa*, the $\sigma^E$ factor (AlgU) is essential for alginate production. In both bacteria, the activity of this sigma factor is regulated by the product of the *mucA*, *mucB*, *mucC*, and *mucD* genes. In this work, we studied the transcriptional regulation of the *A. vinelandii algU-mucABCD* gene cluster, as well as the role of the *mucA* and *mucC* gene products in alginate production. Our results show the existence of AlgU autoregulation and show that both MucA and MucC play a negative role in alginate production.**

*Azotobacter vinelandii* is a gram-negative soil bacterium which under adverse environmental conditions undergoes a differentiation process leading to the formation of desiccation-resistant cysts (37). The mature cysts are surrounded by two capsule-like layers containing a high proportion of the exopolysaccharide alginate (40). This exopolysaccharide is essential for the encystment process, since nonmucoid strains fail to encyst (6, 31, 34).

Considerable information about alginate biosynthesis and its regulation is available based on the studies of *Pseudomonas aeruginosa* (4, 5, 11, 12, 16, 19, 27, 28, 42–46). The interest in this bacterium is motivated by the role that alginate plays in the pathogenesis of the lung 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 (17). The alginate biosynthetic pathways are very similar in *A. vinelandii* (38) and *P. aeruginosa* (28).

In *A. vinelandii*, as in *P. aeruginosa*, the *algD* gene, coding 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 (28). In *A. vinelandii*, the biosynthetic gene cluster is arranged in three operons, one of which transcribes the *algD* gene alone (6, 24, 30), while in the latter bacterium, this gene cluster is transcribed as a single operon, whose transcription is started from a promoter upstream of the *algD* gene (9). The alternative sigma factor $\sigma^E$ (also known as AlgU or AlgT) is responsible for the transcription of the *algD* gene in *P. aeruginosa* (44), as well as in *Pseudomonas syringae* (21). In *A. vinelandii*, *algD* is transcribed from three promoters, only one of which is $\sigma^E$ dependent (34), but *algU* mutants are completely abrogated in alginate production (26, 34), presumably due to the $\sigma^E$ dependence of other genes involved in exopolysaccharide synthesis.

In different bacterial species, the alternative sigma factor $\sigma^E$ regulates the expression of functions related to the extracytoplasmic compartments (33). This sigma factor is similar to the *Escherichia coli* and *Salmonella enterica* serovar Typhimurium $\sigma^E$ protein (3, 10, 20, 29, 39). In *E. coli*, the $\sigma^E$ factor is absolutely required for growth at high temperatures (13, 14). In *E. coli*, genes encoding the heat shock proteins are transcribed by RNA polymerase holoenzyme containing the alternative sigma factor $\sigma^{32}$, encoded by the *rpoH* gene. At 30°C, the *rpoH* transcripts originate from two promoters, p1 and p2, which are recognized by $\sigma^{70}$ RNA polymerase (13). At 42°C or higher temperatures, almost all transcription of *rpoH* comes from the p3 promoter, which is $\sigma^E$ dependent (13, 14).

In *P. aeruginosa* and *A. vinelandii*, the *mucABCD* genes are located downstream of the *algU* gene, forming part of the same transcriptional unit (26, 43). It has been clearly shown elsewhere for *P. aeruginosa* that the AlgU activity is negatively regulated by the anti-sigma factor MucA (11, 12, 16, 19, 27, 43, 45) and, in an indirect manner, by MucB (27) and also that the *mucD* gene encodes a periplasmic protease which plays a central role in AlgU activation (4). MucC has been shown to play a role in AlgU regulation for *P. aeruginosa*, but its mechanism has not been elucidated (5). For *Photobacterium* strain SS9, a gene cluster carrying homologs of *algU*, *mucA*, *mucB*, and *mucC* has been described elsewhere (8). The *mucC* homolog (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 (8). In serovar Typhimurium, the gene homologous to *mucC* has been shown to be involved in biotin synthesis (3). It has been reported that alginate production in *P. syringae* is also regulated by the AlgU-MucA sigma factor–anti-sigma factor (21).

*P. aeruginosa* AlgU and *E. coli* $\sigma^E$ are interchangeable in the *P. aeruginosa* background (46). The *E. coli* chromosome contains, downstream of *rpoE*, two genes (*rseA* and *rseB*) encoding proteins with regulatory functions similar to those of MucA and MucB proteins (10). In *A. vinelandii* (26), the genetic arrangement of *algU-mucABCD* is the same as in *P. aeruginosa* (43), showing high sequence similarity (26). We have previously reported that the *A. vinelandii* and *P. aeruginosa algU-mucABCD* gene products play similar regulatory roles in alginate biosynthesis since they are functionally interchangeable (26). It is also clear that in *A. vinelandii* AlgU is absolutely

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TABLE 1. Bacterial strains and plasmids used in this work

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| Strains | | |
| *A. vinelandii* | | |
| AEIV | Wild type, mucoid | Svein Valla |
| AEA8 | AEIV with a nonpolar *mucA*::Gm mutation | This work |
| AEA4 | AEIV with a polar *mucA*::Gm mutation | This work |
| AEC2 | AEIV with a nonpolar *mucC*::Tc mutation | This work |
| ATCC 9046 | Highly mucoid due to the *muc-1* spontaneous mutation | 26 |
| JRA8 | ATCC 9046 with a nonpolar *mucA*::Gm mutation | This work |
| JRA4 | ATCC 9046 with a polar *mucA*::Gm mutation | This work |
| MLC2 | ATCC 9046 with a nonpolar *mucC*::Tc mutation | This work |
| MLC4 | ATCC 9046 with a polar *mucC*::Tc mutation | This work |
| WI12 | *algD*::*lacZ* derivative of ATCC 9046 | 6 |
| WIA8 | WI12 with a nonpolar *mucA*::Gm mutation | This work |
| WIA4 | WI12 with a polar *mucA*::Gm mutation | This work |
| WIC2 | WI12 with a nonpolar *mucC*::Tc mutation | This work |
| WIC4 | WI12 with a polar *mucC*::Tc mutation | This work |
| *E. coli* | | |
| DH5α | *supE44* Δ*lacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | 18 |
| CAG16037 | MC1061 λ(*rpoH* p3-*lacZ*) | 29 |
| CAG22216 | CAG16037; *rpoE*::ΩCm | 39 |
| Plasmids | | |
| pBluescript SK(+) | Plasmid used for subcloning DNA | Stratagene |
| pMOS*Blue* | Plasmid used for subcloning PCR products | Amersham |
| pHP45Ω-Tc | Plasmid used to obtain the $Tc^r$ cassette | 15 |
| pBSL141 | Plasmid used to obtain the $Gm^r$ cassette | 1 |
| pLRA | pMOS*Blue* derivative carrying a 1.3-kb DNA fragment containing *A. vinelandii mucA* gene amplified by PCR | This work |
| pLRA8 | pLRA derivative containing *mucA*::Gm nonpolar mutation | This work |
| pLRA4 | pLRA containing mutation *mucA*::Gm polar to *mucBCD* | This work |
| pLRC | pMOS*Blue* derivative carrying a 1.3-kb DNA fragment containing *A. vinelandii mucC* gene amplified by PCR | This work |
| pLRC2 | pLRC derivative containing *mucC*::Tc nonpolar mutation | This work |
| pLRC4 | pLRC derivative containing mutation *mucC*::Tc polar to *mucD* | This work |
| pJMSAT1 | Plasmid containing *A. vinelandii algU-mucA* genes; Ap | 34 |

required for cyst formation, independently of its role in alginate production (34).

Overproduction of alginate by *P. aeruginosa* is an important virulence determinant expressed by this organism in the lungs of cystic fibrosis patients (17). Although the initial colonizing *P. aeruginosa* strains are nonmucoid, they undergo conversion to a highly mucoid phenotype in later stages of the disease. Loss-of-function mutations in either *mucA* or *mucB* have been reported to convert *P. aeruginosa* to mucoidy, by increasing AlgU activity (11, 12, 25). In contrast, *A. vinelandii* strains produce alginate even in the absence of mutations in the *mucABCD* operon.

In this context, our aim in this work was to evaluate the effect of *mucA* and *mucC* mutations on alginate production by *A. vinelandii* strains producing different exopolysaccharide levels. We show that the transcription of the *A. vinelandii algU* gene is initiated from two AlgU-dependent promoters, one of which presents a consensus sequence for the recognition of RNA polymerase containing a $\sigma^E$ subunit, and an apparently $\sigma^D$ promoter, which seems to be regulated indirectly by AlgU. It is also shown that the *A. vinelandii* AlgU sigma factor is functional in an *E. coli* background, but with a much lower apparent activity than that of the corresponding *E. coli* protein. We also show that in *A. vinelandii* the MucA and MucC proteins negatively regulate alginate production.

## MATERIALS AND METHODS

**Microbiological methods.** Bacterial strains and plasmids used in this work are shown in Table 1. *A. vinelandii* strains were routinely grown on BS medium (22) at 30°C. Antibiotic concentrations used for *A. vinelandii* and *E. coli* were as follows: ampicillin, not used and 200 μg/ml; chloramphenicol, not used and 30 μg/ml; gentamicin, 1.5 and 10 μg/ml; kanamycin, 2 μg/ml and not used; and tetracycline, 20 and 20 μg/ml, respectively.

Triparental or biparental *A. vinelandii* matings were done as reported previously (6). *A. vinelandii* transformation was done as reported by Bali et al. in 1992 (2). Alginate production was measured by the method described previously (23).

β-Galactosidase activity was determined as reported by Miller (32); 1 U corresponds to 1 nmol of *O*-nitrophenyl-β-D-galactosidase hydrolyzed per min and per mg of protein. All measurements were done in triplicate.

**Nucleic acid procedures.** DNA isolation, cloning, and sequencing; Southern blotting; and nick translation procedures were carried out as described previously (41). Primer extension analysis of *A. vinelandii algU* was done with U1 oligonucleotide (5′-CAATTGCTGATCTTGCTCCTGG-3′) located in the 5′ region of this gene. Primer extension of *algD* was carried out as previously described (6), using an Amersham primer extension kit as instructed by the manufacturer. The sequencing reaction shown in the primer extension analysis was done with the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science, Inc.).

**Construction of plasmids pLRA and pLRC.** The *A. vinelandii mucA* and *mucC* genes were amplified by PCR using ATCC 9046 chromosomal DNA as a template as well as oligonucleotides *mucA*- 5′ GGCGAGCCTTCGATTTGCTG and *mucA*-3′ CTGCCGTTACGCTCGTAGA and *mucC*-5′ GTCCTGCCTGCCAA CCTG and *mucC*-3′ GACTGTGGGGAGCATTCG, respectively. The resulting 1,301- and 1,324-nucleotide PCR products were cloned in pMOS*Blue*, producing plasmids pLRA and pLRC, respectively (Table 1 and Fig. 1).

**Construction of polar and nonpolar *mucA*::Gm and *mucC*::Tc mutations.** We have previously reported, by Northern blot hybridization assays, that in *A. vine-*

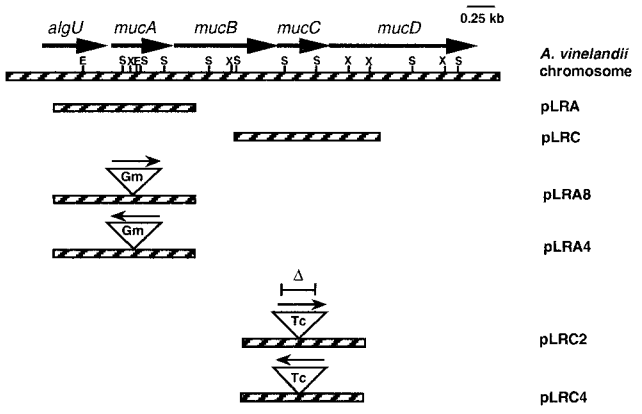



FIG. 1. Physical map of the *algU-mucABCD* region of *A. vinelandii* and of plasmids constructed in this study. Arrows indicate the direction of transcription. Antibiotic resistance cassettes, which are represented by inverted triangles, are not shown to scale. Abbreviations: E, *Eco*RV; S, *Sty*I; X, *Xho*I.

*landii* the insertion of Ω cassettes into genes with the same orientation as the direction of transcription produces nonpolar mutations which allow expression of the downstream genes in the same operon, whereas the insertion of the cassette in the opossite orientation produces a polar mutation (7, 35, 36). This is also the case for the gentamicin cassette gene described previously (1, 36) and used in the present study. Plasmid pLRA was used to introduce into the unique *Xho*I site of *mucA*, a 0.8-kb *Xho*I fragment containing a gentamicin resistance cassette (1). Clone derivatives containing the gentamicin cassette ligated in both orientations were selected, producing plasmids pLRA8, containing a *mucA*::Gm nonpolar mutation (*mucA*), and pLRA4, containing a *mucA*::Gm polar mutation to *mucBCD* (resulting in a *mucABCD* mutation). Plasmid pLRC was cleaved with *Sty*I (releasing a 300-bp DNA fragment of the *ampC* gene), blunt ended, and ligated to a 2.0-kb *Sma*I fragment containing a Ω-tetracycline cassette. Clone derivatives containing the Ω-tetracycline cassette ligated in both orientations were selected, producing plasmids pLRC2, containing a *mucC*::Tc nonpolar mutation (*mucC*), and pLRC4, containing a *mucC*::Tc mutation polar to *mucD* (producing a *mucCD* mutation). Plasmids pLRA8, pLRA4, pLRC2, and pLRC4 (Fig. 1) were unable to replicate in *A. vinelandii* and were used to introduce the *mucA*, *mucABCD*, *mucC*, and *mucCD* mutations into strains ATCC 9046, AEIV, and WI12. Transformants were selected using the corresponding antibiotic and confirmed by Southern blot analysis to carry the desired mutations (Fig. 2).

## RESULTS AND DISCUSSION

**Effect of *mucA*, *mucABCD*, and *mucC* mutations on alginate production in two *A. vinelandii* strains.** To determine the role of MucA, MucB, MucC, and MucD proteins in alginate production by *A. vinelandii*, we constructed derivatives of the wild-type strain AEIV carrying *mucA*, *mucABCD*, and *mucC* mutations as described in Materials and Methods and found that the three mutants present a significant increment of alginate production (Table 2). These results reinforce our previous findings (26), based on the complementation of *P. aeruginosa mucA* mutants, that in *A. vinelandii*, as in *P. aeruginosa*, MucA and possibly MucB and MucD products function as negative regulators of AlgU activity. Thus, the disruption of the *mucA*, *mucB*, or *mucD* gene results in an increase of this sigma factor activity, as has been shown for *E. coli* (10) and *P. aeruginosa* (27), increasing *algD* transcription and possibly that of other *alg* genes and ultimately alginate production. In *A. vinelandii*, however, MucC seems to function directly as a negative regulator of AlgU activity since *mucC* mutants have higher alginate production (Table 2), while in *P. aeruginosa* MucC does not directly affect AlgU activity (5). The mechanism of AlgU activity regulation by MucC in *A. vinelandii* remains to be determined.

The AEIV *mucABCD* mutant (AEA4) presents the most drastic phenotype as evaluated by alginate production on plates (Table 2). However, when alginate production was quantitated on liquid cultures, this mutant did not show the highest increase in alginate production (Table 2). This lack of correlation is due to the high instability of the mutant due to the selection of spontaneous mutants with a reduced alginate production, possibly affecting AlgU expression. This instability is so high that after four subcultures the mutant AEA4 completely loses its increased alginate production. It is also apparent that the higher the alginate production by any of the *muc* mutants, the lower the growth rate of the strain (data not shown).

Strain ATCC 9046 is highly mucoid, due to the presence of a spontaneous regulatory mutation, called *muc-1*, which upregulates AlgU activity (26). The effect of the mutations on the

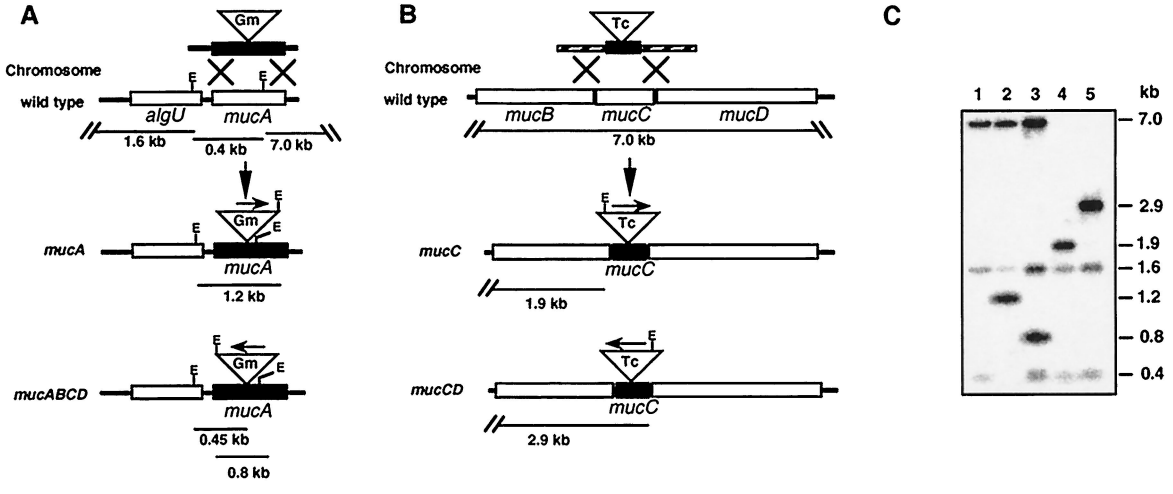



FIG. 2. Schematic representation of the strategy followed to construct *mucA* and *mucC* mutants. (A and B) Insertional inactivation of the *mucA* (A) and *mucC* (B) genes producing the respective polar and nonpolar mutations. (C) Southern blot hybridization of total genomic DNA digested with *Eco*RV endonuclease with plasmid pRLA4 as a probe. Lanes: 1, ATCC 9046; 2, JRA8 (*mucA*); 3, JRA4 (*mucABCD*); 4, MLC2 (*mucC*); 5, MLC4 (*mucCD*). Identical hybridization patterns were found for strain AEIV and its corresponding *muc* mutants (data not shown).

TABLE 2. Alginate production in different *A. vinelandii* strains

| Strain | Genotype | Mean alginate concn[a] (mg/mg of protein) ± SEM (%) | Mucoidy[b] |
|---|---|---|---|
| AEIV | Wild type | 0.9 ± 0.2 (100) | + |
| AEA8 | AEIV, *mucA* | 5.9 ± 0.4 (651) | +++ |
| AEA4 | AEIV, *mucABCD* | 4.0 ± 0.15 (444) | ++++ |
| AEC2 | AEIV, *mucC* | 2.4 ± 0.4 (266) | ++ |
| ATCC 9046 | Wild type, *muc-1* | 4.8 ± 0.3 (100) | +++ |
| JRA8 | ATCC 9046, *mucA* | 6.7 ± 0.8 (139) | +++ |
| JRA4 | ATCC 9046, *mucABCD* | 8.9 ± 0.7 (185) | +++++ |
| MLC2 | ATCC 9046, *mucC* | 4.0 ± 0.3 (83) | +++ |
| MLC4 | ATCC 9046, *mucCD* | 5.1 ± 0.2 (106) | +++ |

[a] Alginate was determined in cells grown for 48 h on liquid Burke's nitrogen-free salts supplemented with 2% sucrose as a carbon source.
[b] + to +++++, least to most mucoidy, respectively.

*muc* genes is different in the highly mucoid strain ATCC 9046, since neither *mucC* nor *mucCD* mutations increased alginate production (Table 2). The different response of the two studied strains is very probably due to a high basal level of AlgU activity in strain ATCC 9046, caused by the *muc-1* mutation (26).

An increase of approximately twofold was observed in the ATCC 9046 *mucABCD* mutant strain JRA4, giving the *A. vinelandii* strain the highest specific alginate production, to our knowledge. The selection of spontaneous mutations that presented a reduced level of alginate production (2.5 mg/mg of protein) was apparent in mutant JRA4 as well as in mutant AEA4.

The ATCC 9046 *mucA* nonpolar mutant (JRA8) showed a low, but significant, increase of alginate production (Table 2). It is apparent from this result that in *A. vinelandii* MucA by itself plays an important role in the negative regulation of AlgU activity, even in a strain with elevated basal AlgU activity (26). The difference in levels of alginate production between mutants JRA4 (*mucABCD*) and JRA8 (*mucA*) show that MucB, MucC, and/or MucD affects AlgU activity by a different signaling mechanism than that of the anti-sigma factor MucA.

Even though mutant JRA8 presented a considerably lower increase in alginate production than that of mutant JRA4, the former mutant is also unstable with respect to hyperproduction of alginate. The instability of these mutants suggests that the elevated AlgU activity or the increased alginate production might be deleterious to *A. vinelandii*.

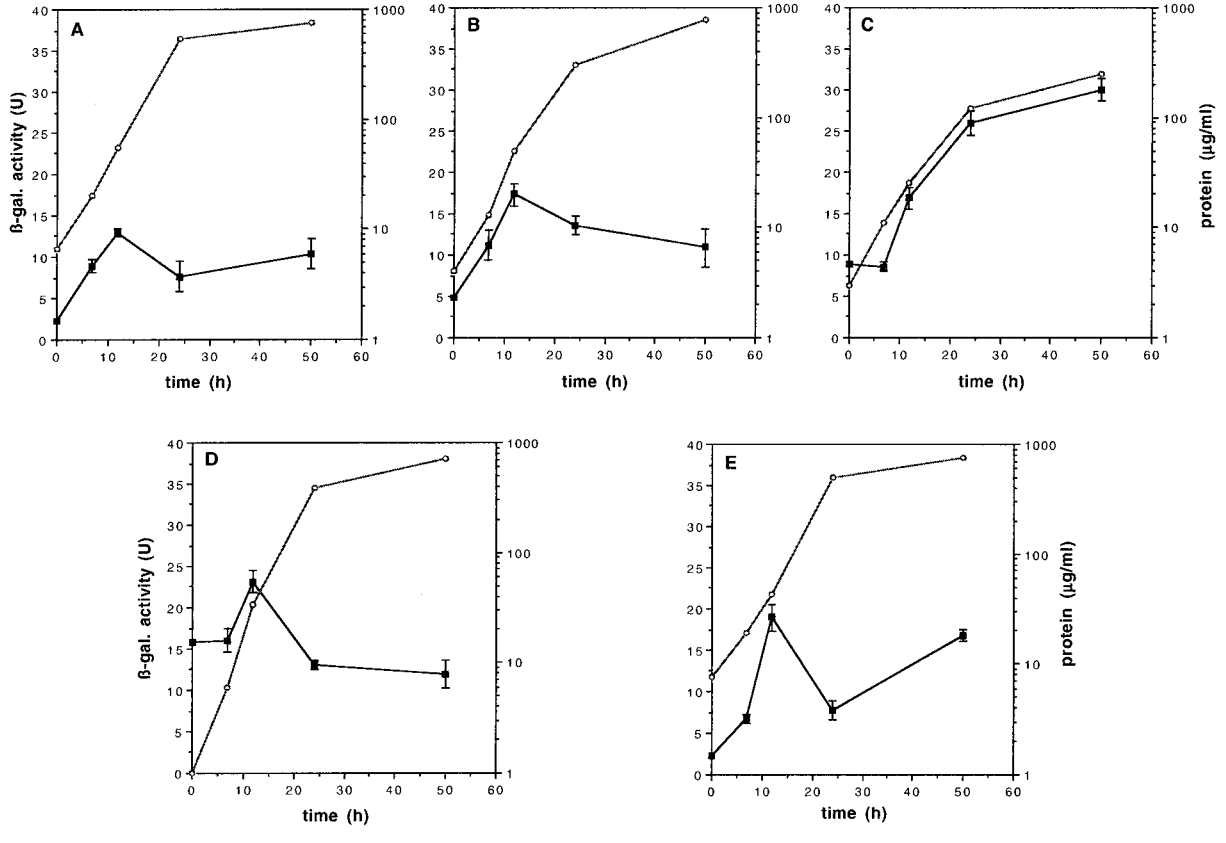



FIG. 3. Growth (open symbols) and β-galactosidase activity (closed symbols), on Burke's medium supplemented with 2% sucrose, of strains. WI12 (parental strain) (A), WIA8 (*mucA*) (B), WIA4 (*mucABCD*) (C), WIC2 (*mucC*) (D), and WIC4 (*mucCD*) (E).

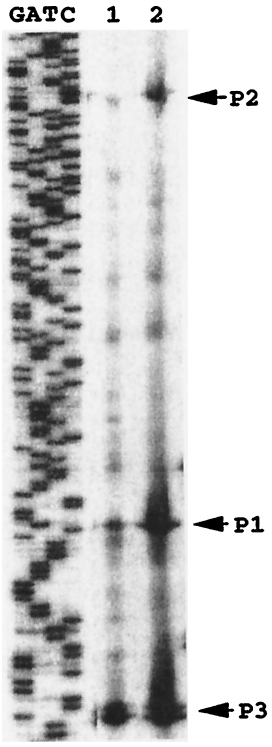
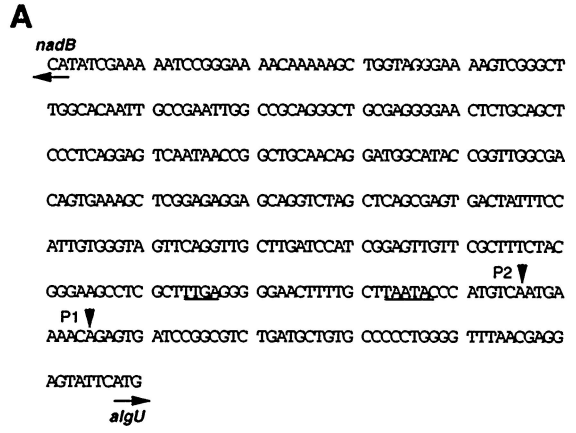
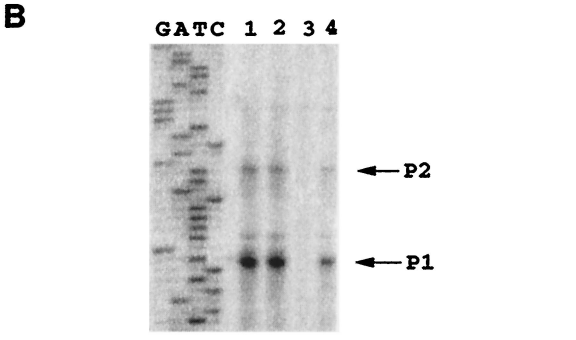



FIG. 4. Primer extension analysis of *algD* transcription. Lanes correspond to RNA extracted from strain ATCC 9046 (lane 1) and JRA4 (lane 2). Each reaction contained as template 50 μg of RNA isolated from bacterial cultures grown for 48 h in Burke's medium supplemented with 2% sucrose.



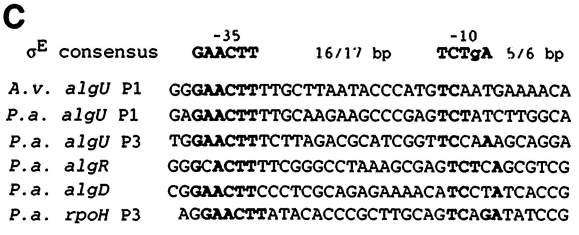




FIG. 5. Primer extension analysis of *algU* transcription. (A) DNA sequence of the 5′ region of *algU*. p1 and p2 mRNA initiation sites are indicated. The −10 and −35 regions of the p2 promoter are underlined. (B) Primer extension analysis of the *algU* gene. Lanes correspond to RNA extracted from the following strains: ATCC 9046 (lane 1), JRA4 (*mucABCD*) (lane 2), SMU88 (*algU*) (lane 3), and AEIV (lane 4). (C) Sequence alignment of several AlgU ($\sigma^E$)-dependent promoters. Abbreviations: *P.a.*, *P. aeruginosa*; *A.v.*, *A. vinelandii*.

**Effect of *mucA*, *mucABCD*, and *mucC* mutations on *algD* transcription.** Most of the molecular genetics analysis of alginate production in *A. vinelandii* has been carried out in the highly mucoid strain ATCC 9046 (6, 7, 24, 26, 30, 31, 34, 35, 36). The detailed analysis of the structure of the regulatory region of the AEIV *algD* gene is currently being performed. At present, we have only preliminary evidence suggesting that the regulatory elements participating in AEIV *algD* transcriptional regulation are the same as those involved in the regulation of this gene in strain ATCC 9046 (6, 36) and that the main difference between the strains seems to be the high AlgU activity in the latter strain due to an uncharacterized *muc-1* mutation (26). In order to further characterize the effect of the inactivation of the *muc* genes on the *algD* transcriptional regulation, we focused our research on strain ATCC 9046 and its derivatives.

To evaluate the effect of the polar and nonpolar *mucA* and *mucC* mutations on *algD* transcription, they were transferred, as described in Materials and Methods, to strain WI12, an ATCC 9046 derivative which carries an *algD-lacZ* transcriptional fusion. We have previously reported that in strain WI12 the transcription of *algD* increased during early exponential phase and declines in prestationary phase (7). As shown in Fig. 3, *mucA* and *mucC* mutations increased *algD* transcription from one- to twofold along the entire growth curve, with a kinetics similar to that observed in the parental strain WI12, whereas the *mucABCD* mutant shows an approximately fourfold increase of *algD* transcription during the stationary phase of growth. A deregulation of *algD* transcription along the entire growth curve was observed in the WIA4 strain, which carries a *mucABCD* mutation, even though the maximum upregulation of *algD* transcription was observed during stationary phase (Fig. 3C). The increased *algD* expression in the ATCC 9046 *mucC* and *mucCD* mutant background (mutants WIC2 and WIC4 [Fig. 3D and E]) may indicate that in this highly mucoid strain MucC also exerts a direct negative role on AlgU activity, even though this effect is not so strong as to be reflected in the amount of alginate produced by the mutants.

Mutant WIA4 (*mucABCD*) presented the highest increase in *algD* expression (Fig. 3C), in accordance with the highest alginate production being that of mutant JRA4 (*mucABCD*). The pronounced increase in *algD* expression for WIA4, in contrast to the lower increase observed in mutant WIA8 (Fig. 3B), further supports the involvement of MucB, MucC, and/or MucD in a signaling cascade that affects AlgU activity through a different route from that of MucA.

The *A. vinelandii* ATCC 9046 *algD* gene is transcribed from three promoters: p1, a $\sigma^D$ promoter; p2, an AlgU ($\sigma^E$)-dependent promoter; and a p3 promoter which shows no recognized

TABLE 3. Determination of *A. vinelandii* AlgU activity in the *E. coli* background

| Strain | Genotype | β-Galactosidase (U)[a] at min: | | |
|---|---|---|---|---|
| | | 0 | 30 | 60 |
| CAG16037 | *rpoH* p3::*lacZ* | 8.75 | 10.8 | 9.17 |
| CAG22216 | *rpoE*::Cm$^r$ *rpoH* p3::*lacZ* | ND | ND | ND |
| CAG22216/pJMSAT1 | *A. vinelandii algU*$^+$ *mucA*$^+$ | 1.12 | 1.6 | 2.0 |

[a] β-Galactosidase activity reflects the level of *E. coli rpoH* p3 promoter which is fully dependent on $\sigma^E$ activity and was determined in cultures after shift to 42°C for the indicated time in minutes. Cells were grown on M9 medium as described previously (29). The data presented are the averages of three independent experiments. ND, not detected.

consensus sequences (6, 34, 35). It thus seemed likely that the absence of the Muc products in the *mucABCD* mutant would upregulate AlgU activity, which in turn would increase *algD* transcription from the p2 promoter. To verify this hypothesis, primer extension analysis of the *algD* gene was carried out on the *mucABCD* mutant. As shown in Fig. 4, *mucABCD* mutation increased *algD* transcription from its three promoters and not only from the p2 AlgU-dependent promoter. We have previously reported that an *ampDE* mutation in the ATCC 9046 background resulted in an increased *algD* initiation of transcription from its three promoters (36) and that an ATCC 9046 *gacS* mutant presented a decreased transcription from the three *algD* promoters (7). These results, together with the data presented here, strongly suggest the existence of a common level of regulation of the three *algD* promoters.

**Transcriptional regulation of the *algU* gene.** In order to investigate the nature and regulation of the *A. vinelandii* sigma factor AlgU, primer extension analysis of the *algU* gene was carried out on strains AEIV and ATCC 9046. As shown in Fig. 5B, in both strains there is a putative transcriptional start site (p1) located 54 nucleotides upstream of the ATG start codon and another putative promoter (p2) starting transcription 62 nucleotides upstream of the translational start site. As shown in Fig. 5C, the p1 −10 and −35 DNA sequences correspond very well to the $\sigma^E$-dependent promoter consensus sequences. The −10 and −35 p2 sequences suggest that this is a $\sigma^D$ promoter (Fig. 5A).

To further characterize these promoters, primer extension analysis was carried out using RNA derived from ATCC 9046 *algU* mutant SMU88 (34). As expected, the primer extension product corresponding to p1 was not observed in strain SMU88, confirming the dependence of this promoter on the AlgU sigma factor. Unexpectedly, the p2 start site was also abrogated, suggesting that the transcription from this promoter is also regulated by AlgU, but in an indirect manner. These results thus show that *algU* transcription is autoregulated, as it is in *P. aeruginosa* (42). All genes which are activated by autoregulation need a constitutive promoter to maintain a basal level of expression; we could not detect the promoter responsible for the basal expression of the *algU-mucABCD* operon.

We reported previously that the ATCC 9046 *algD* p2 promoter was AlgU dependent based on its lack of expression on an *algU* mutant (34) and on the presence of sequences in the −10 and −35 regions showing a reduced similarity with the $\sigma^E$ consensus sequences (6). The *algD* p2 promoter was the first reported AlgU-dependent promoter in *A. vinelandii*, and so there was no other sequence for comparison and validation of the significance of the detected homology. The high sequence similarity of *algU* p1 with $\sigma^E$-dependent promoters from different bacteria (Fig. 5C) strongly suggests that the *algD* p2 promoter is not directly recognized by AlgU.

Initiation of *algU* transcription in the ATCC 9046 background seems to be much more frequent from the p1 promoter than from the p2 initiation site, whereas in strain AEIV both initiation sites show the same intensity (Fig. 5B). These results suggest that the *muc-1* mutation present in strain ATCC 9046 increases AlgU activity by increasing *algU* transcription from p1, the promoter directly recognized by AlgU itself.

The finding of AlgU autoregulation and the increased *algU* transcription from the p1 promoter in strain ATCC 9046 suggested to us that the different *muc* mutants might show an increased level of *algU* transcription. However, we found that, in both strains studied, all the *muc* mutants showed similar levels of *algU* transcription (see Fig. 5B for an example). These results further reinforce our previous findings suggesting that, as in *P. aeruginosa mucA* and *mucB* (27), MucA, MucB, MucC, and MucD modulate AlgU activity and not the transcription of the *algU* gene. In contrast, the *E. coli rseA* mutants present a 12-fold increase in *algU* transcription (10).

**Activation of *rpoH* p3 initiation of transcription in *E. coli* by *A. vinelandii* AlgU.** In order to test whether *A. vinelandii* AlgU was able to activate the *rpoH* p3 promoter of *E. coli*, plasmid pJMSAT1, which carries the ATCC 9046 *A. vinelandii algU-mucA* genes (34), was transferred by transformation to *E. coli* strains CAG16037 (*rpoH* p3::*lacZ*) and CAG22216 (*rpoE*::Cm$^r$, *rpoH* p3::*lacZ*), and the effect of a heat shock treatment (30 to 42°C) was evaluated by measuring the kinetics of β-galactosidase expression (Table 3). *A. vinelandii* AlgU restored from 12 to 20% of the *rpoH* p3 transcription in *E. coli* (Table 3). This reduced level of expression is sufficient to complement the CAG22216 ability to grow on plates at 42°C (data not shown).

We have thus shown that *A. vinelandii* AlgU is able to complement an *E. coli rpoE* mutant for growth on plates at 42°C. This result shows that the function of both proteins in transcription at high temperatures is conserved, at least partially. However, activity of *A. vinelandii* AlgU in the *E. coli* background accounts for only around 15% of the detected activity of the *E. coli* $\sigma^E$ factor in the transcription from *rpoH* p3 (Table 3). The rate-limiting step for this reduced AlgU activity, whether at the level of expression or the level of protein function, of the *A. vinelandii* AlgU activity in the *E. coli* background remains to be determined.

We have previously reported evidence showing that in *A. vinelandii* AlgU activity is regulated by the *muc* gene products in a manner similar to that reported previously for *P. aeruginosa* (26). These data, together with the increase in alginate production and *algD* expression in *mucA* and *mucABCD* mutants reported here, suggest that, in *A. vinelandii*, AlgU activity is negatively regulated by MucA and possibly also by MucB and MucD. On the other hand, our results show that MucC is by itself a negative regulator of alginate production in *A. vinelandii* (Table 2), contradicting the previously reported results on the lack of a direct effect of MucC in *P. aeruginosa* (5). The mechanism of AlgU regulation by MucC in *A. vinelandii* is presently unknown.

The disruption of any of the *muc* genes did not affect encystment frequencies or cyst morphology of the two strains studied, AEIV and ATCC 9046 (data not shown). These data show that the level of alginate production does not correlate with the proportion of cells undergoing differentiation and suggest that AlgU activity is not the rate-limiting step in cyst formation. The same two conclusions were attained when cyst formation was evaluated on strains with reduced levels of AlgU expression (34).

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