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Characterization of the Gene Coding for GDP-Mannose Dehydrogenase (*algD*) from *Azotobacter vinelandii*

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Azotobacter vinelandii **presents a differentiation process leading to the formation of desiccation-resistant cysts. Alginate, the exopolysaccharide produced by this bacterium, has been postulated to have a role in cyst formation. Here, we report the cloning and characterization of the *A. vinelandii* gene coding for the enzyme GDP-mannose dehydrogenase (*algD*), which is the key enzyme for alginate synthesis in *Pseudomonas aeruginosa*. This gene has a high degree of similarity with the *algD* gene from *P. aeruginosa*, and similar proteins seem to be involved in *algD* regulation in both bacteria. We show the existence of two mRNA start sites; one of these sites corresponds to a promoter transcribed by RNA polymerase containing a σ^E subunit. An *A. vinelandii algD* mutant which is completely impaired in alginate production and which is unable to form desiccation-resistant cells was constructed. The effects of NH_4, NO_3, and NaCl concentrations on *algD* transcription for three *A. vinelandii* strains producing different alginate levels were evaluated. We found a strict correlation between alginate production and *algD* transcription for the three strains studied; however, the effects on *algD* transcription under the conditions studied were different for each strain. The nitrogen source regulates *algD* expression in the wild-type strain.**

Azotobacter vinelandii is a gram-negative soil bacterium which suffers a differentiation process leading to the formation of desiccation-resistant cysts under adverse environmental conditions. The encystment process takes approximately 5 days, and the mature cysts are surrounded by two capsule-like layers containing a high proportion of alginate (19, 35, 44); these dormant cells are able to survive in dry soil for long periods. Other particular features of this bacterium are that under some conditions it has multiple copies of its entire chromosome (24, 26, 34) and that it is capable of fixing nitrogen under aerobic conditions (21).

Alginate is a linear polysaccharide constituted by mannuronic and guluronic acids. It is able to form heat-resistant gels in the presence of Ca^{2+}; this property makes this biopolymer useful for different industrial applications (42). The alginate used in industry is extracted from marine brown algae (42).

Considerable information about alginate biosynthesis and regulation in *Pseudomonas aeruginosa* has been described elsewhere (4, 29). These studies are motivated by the pathogenesis of this bacterium: lung infections with *P. aeruginosa* mucoid strains, which produce high amounts of alginate, are the most important cause of death of cystic fibrosis patients.

The alginate biosynthetic pathways from *A. vinelandii* and *P. aeruginosa* are very similar (4, 37). In *P. aeruginosa*, the rate-limiting enzyme in the pathway is GDP-mannose dehydrogenase, which converts GDP-mannose, a metabolite used for the synthesis of different saccharides, to GDP-mannuronic acid, a direct alginate precursor. The gene coding for GDP-mannose dehydrogenase is called *algD* (4, 29, 40).

In *P. aeruginosa*, *algD* is located in a biosynthetic cluster which contains most of the genes coding for the enzymes involved in alginate synthesis. The gene cluster appears to be transcribed as an operon (5), which is controlled from the *algD* promoter via positive transcriptional regulation by the proteins AlgR and AlgB (8, 15, 16, 31). The recently identified sigma factor AlgU is responsible for the initiation of *algD* and *algR* and its own transcription (18, 27, 43). AlgU activity is negatively regulated by the products of *mucA* and *mucB*; this sigma-like factor is similar to the *Escherichia coli* σ^E protein, and both recognize similar DNA sequences (11, 12, 18, 39).

The native *P. aeruginosa* strains isolated from environmental samples or from some infected human tissues do not produce alginate at significant levels. The lack of alginate biosynthesis is due to a very low level of AlgU activity, which is inactivated by MucA and MucB. All of the naturally occurring mucoid strains are isolated from the lungs of cystic fibrosis patients and contain mutations either in *mucA* or in other not yet identified loci (11, 12, 14, 39). At present, there is not a clear picture of the physiological role alginate biosynthesis might play for *P. aeruginosa* in the environment (29).

A. vinelandii has been reported to contain DNA sequences homologous to those of some *P. aeruginosa* genes involved in the biosynthesis and regulation of alginate production, such as *algD* and *algR* (13).

In *A. vinelandii*, alginate has been proposed to play an important structural role in cysts (44); however, most of the isolates produce this exopolysaccharide also during vegetative growth. Alginate production in *A. vinelandii* is thus a unique biological model, and its study at the molecular level will help to answer different questions of biological importance.

In this paper, we report the cloning and characterization of

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristic(s)	Source or reference
A. vinelandii		
ATCC 9046	Highly mucoid	Collection
UW	Nonmucoid	36
UW136	Rif^r derivative of UW	20
AEIV	Mucoid	Svein Valla
RSD1	*algD* mutant derived from ATCC 9046	This work
WI12	ATCC 9046 with a mini-Tn*5lacZ1* insertion in *algD*	This work
U5	UW136 with a mini-Tn*5lacZ1* insertion in *algD*	This work
A2	AEIV with a mini-Tn*5lacZ1* insertion in *algD*	This work
E. coli		
S17.1	*thi pro hsd* (r^- m^+) *recA*::RP4-2-Tc^r::Mu Km^r::Tn*7* Tp^r Sm^r	41
S17.1 (λpir)	Same as S17.1, but expressing λpir protein and thus able to replicate pUT plasmids	7
DH5α	*supE44* Δ*lacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	17
Plasmids		
pCP13	RK2-derived cosmid vector; Tc^r Km^r	6
pMSD675	Plasmid containing 25 kb of *A. vinelandii* DNA including *algD*, derived from pCP13	This work
pKOK4	pBR325-derived *mob*$^+$ plasmid; Tc^r Ap^r Cm^r	23
pMSD27	Plasmid containing 5.5 kb of *A. vinelandii* DNA including *algD*, derived from pKOK4	This work
pCTD273	Derivative of plasmid pMSD27 with a mini-Tn*5lacZ1* insertion on the *algD* gene	This work
pBluescript SK$^+$	Plasmid used for subcloning DNA to be sequenced; Ap^r	Stratagene
pMSDX7	Plasmid containing 594 bp of the *A. vinelandii algD* gene coding from amino acids 137 to 337, derived from pSK$^+$	This work
pUT mini-Tn*5lacZ1*	Suicide vector for mutagenesis with mini-Tn*5lacZ1*; able to replicate only in strains expressing λpir protein; Km^r	7

the *A. vinelandii algD* gene, including its sequence analysis; we show the effect of its mutagenesis on the formation of desiccation-resistant cells and the effects of different culture conditions on its transcription in three strains.

MATERIALS AND METHODS

Microbiological procedures. The bacterial strains and plasmids used in this work are shown in Table 1. *A. vinelandii* strains were routinely grown on BS medium, which lacks a fixed source of nitrogen (20), at 30°C. Modified BS medium was used for the experiment shown in Fig. 5 and contains (in grams per liter) K_2HPO_4 (0.66), KH_2PO_4 (0.16), NaCl (0.2), $MgSO_4 \cdot 7H_2O$ (0.2), Na_2MoO_4 (0.003), $FeSO_4 \cdot 7H_2O$ (0.274), yeast extract (3.0), morpholinepropanesulfonic acid (MOPS; 1.42), and sucrose (20.0). *E. coli* strains were grown on LB medium (30) at 37°C. Antibiotic concentrations used for *A. vinelandii* and *E. coli*, respectively (in micrograms per milliliter), were 30 and 200 for ampicillin, 30 and 30 for chloramphenicol, 10 and 60 for kanamycin, not used and 30 for nalidixic acid, 10 and not used for rifampin, and 10 and 30 for tetracycline.

Triparental or biparental *A. vinelandii* matings were done as reported previously (20). *A. vinelandii* transformation was done as reported by Bali et al. in 1992 (1).

Alginate production was measured by the spectrophotometric determination of uronic acid (3, 22) or by measuring its dry weight from liquid cultures. All measurements were done in triplicate.

β-Galactosidase activities were determined as reported by Miller (30), with 1 U corresponding to 1 nM *o*-nitrophenyl-β-D-galactoside hydrolyzed per min per mg of protein. All measurements were done in triplicate.

Resistance to desiccation was measured with bacterial cultures grown for 18 to 24 h on liquid BS medium which were transferred to BS plates with 0.2% *n*-butanol as a carbon source, and the plates were incubated for 5 days. Approximately 10^6 CFU of each strain was applied to Millipore 0.2-μm-pore-size membranes and placed in sterile petri dishes, and the dishes were incubated for 5 more days. Dried cells (cysts) were suspended in 1 ml of BS medium, and viable counts were made to determine their number.

Genetic manipulations. The *A. vinelandii algD* mutant was constructed in the ATCC 9046 strain by selection of single recombinant events with plasmid pMSDX7 (Table 1). Plasmid pMSDX7 has a 594-bp *Xho*I fragment containing an internal sequence from the *A. vinelandii algD* gene and is unable to replicate in *Azotobacter* species. This plasmid was introduced into *A. vinelandii* by transformation. The Ap^r clones selected were expected to be the product of a single recombination event that would render two truncated *algD* genes, one devoid of the 5′ end and the other lacking the 3′ end of this gene, separated by the cloning vector pBluescript SK$^+$. One of the Ap^r transformants is strain RSD1 (see Fig. 3), which is presented herein.

The *algD-lacZ* transcriptional fusion was isolated by selecting mini-Tn*5lacZ1* insertions into the *algD* gene located in plasmid pMSD27 (Table 1) by selecting Km^r Nal^r transconjugants from the cross between *E. coli* strains S17.1 (λpir) (containing plasmid pUT mini-Tn*5lacZ1* [7]) and DH5α (containing plasmid pMSD27 [Table 1]); insertions on the plasmid were identified later. Plasmid pCTD273 (Table 1) was shown to contain a mini-Tn*5lacZ1* insertion in the 3′ end and in the same orientation as *algD* (see Fig. 4). The *algD-lacZ* transcriptional fusions in three *A. vinelandii* strains were constructed with the pCTD273 plasmid. Plasmid pCTD273 was cut with *Pst*I endonuclease to obtain only double recombinants; the clones were selected by their Km^r and Ap^s phenotypes after transformation.

To simultaneously measure *algD* transcription and alginate production, plasmid pMSD675 was introduced by conjugation from *E. coli* S17.1 (Table 1) to the *A. vinelandii* strains WI12, U5, and A2 (Table 1), which carry the *algD-lacZ* fusion; the transconjugants were selected on BS medium supplemented with tetracycline and kanamycin.

Total DNA library construction. Total genomic DNA from *A. vinelandii* ATCC 9046 was partially digested with *Sau*3A and was ligated to cosmid pCP13 (6) digested with *Bam*HI endonuclease. Cosmids were packaged onto λ heads and transfected as described previously (25). 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 (25). The *P. aeruginosa algD* gene fragment used as a probe to screen the *A. vinelandii* genomic library has also been described previously (13) and was kindly provided by A. M. Chakrabarty from the University of Illinois at Chicago. DNA sequencing was done either with the Sequenase kit or the *Taq* polymerase enzyme (U.S. Biochemicals) according to the manufacturer's instructions. DNA regions to be sequenced were subcloned in plasmid pBluescript SK$^+$ (Stratagene Cloning Systems). The *algD* mRNA start site was determined by primer extension (25) of RNA extracted from strains ATCC 9046 and UW136, which were grown for 48 h in BS medium, with the oligonucleotide underlined in Fig. 1 used as the primer.

Nucleotide sequence accession number. The nucleotide sequence of the *A. vinelandii algD* gene reported here has been deposited in the GenBank database under accession number U11240.

RESULTS

Cloning and sequencing of the *A. vinelandii algD* gene. It has been previously reported that *A. vinelandii* has DNA sequences

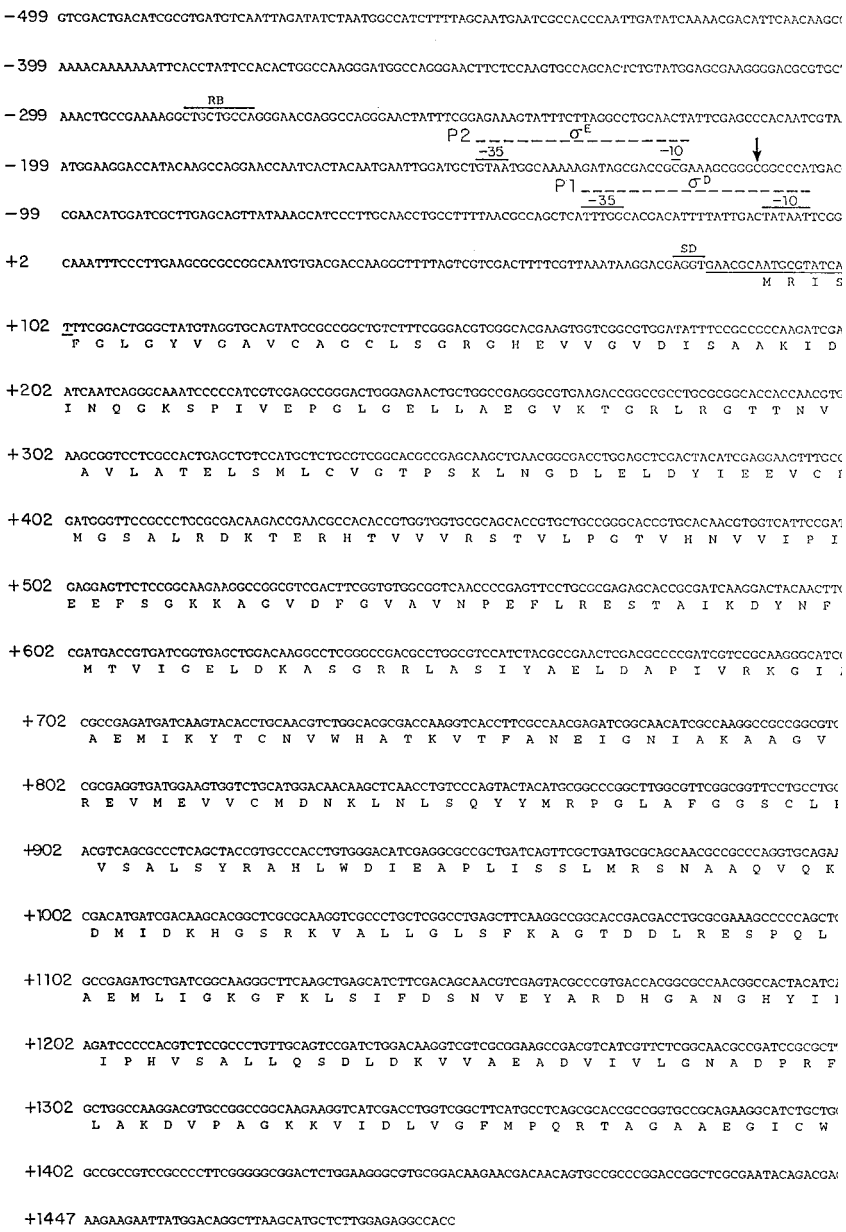

FIG. 1. Nucleotide sequence of the *A. vinelandii* ATCC 9046 *algD* gene and its predicted amino acid coding sequence. The presumptive AlgR binding site (RB) is shown. The oligonucleotide used as primer for the identification of the mRNA start site is underlined. Arrows show the identified mRNA start sites which define P1 and P2 promoters. The first nucleotide of the mRNA transcribed from the P1 promoter is designated +1.

homologous to those of *P. aeruginosa* genes of the alginate biosynthetic pathway (13). The *A. vinelandii algD* gene was cloned from strain ATCC 9046 on the basis of its homology to the corresponding *P. aeruginosa* gene. Southern blot analysis with an internal fragment of the *P. aeruginosa algD* gene as a probe led to the identification of six cosmids carrying a 6-kb *Pst*I fragment with *algD*-homologous sequences. One of them was selected for further analysis (pMSD675). The 6-kb *Pst*I fragment from this plasmid was subcloned in plasmid pKOK4, yielding plasmid pMSD27 (Table 1).

The *A. vinelandii algD* sequence is shown in Fig. 1; it codes for a 47.9-kDa polypeptide, presenting 79% identity at the DNA level and 73% identity at the protein level to its *P. aeruginosa* counterpart (Fig. 2).

Analysis of the DNA sequence upstream from the first ATG led to the identification of some putative transcription regulatory sequences. The promoters recognized by the RNA polymerase (RNAP) containing the σ^E-like subunit, which includes the AlgU protein, recognize the consensus sequence GAAC $TN_{19}TCT$ (12, 18) (underlined nucleotides are invariable); we found two sequences which matched very well with it but which were not functional as judged by the primer extension analysis described below.

AlgU-dependent transcription of the *P. aeruginosa algD* gene is positively regulated by the AlgR protein. Four sequences which this protein binds have been identified in the *algD* upstream region; one of them (RB1) is in the direction opposite to that of the other three (31, 32). We found the

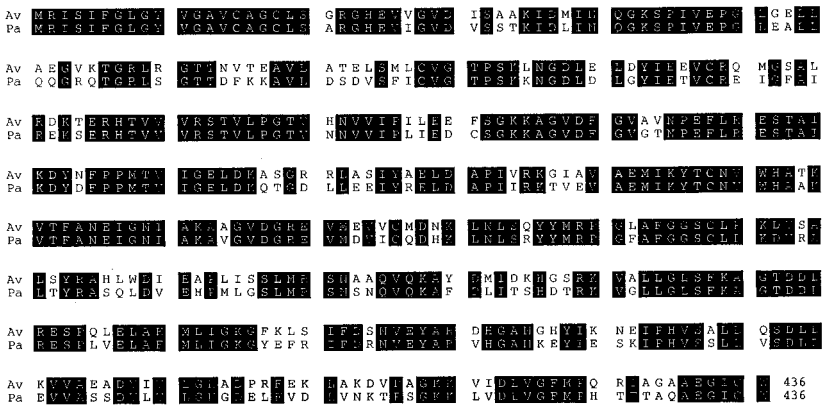

FIG. 2. Alignment of the predicted amino acid AlgD sequence from *A. vinelandii* (Av) and its homolog from *P. aeruginosa* (Pa) (accession number P11579). The alignment was done with the PILEUP program of the University of Wisconsin Genetics Computer Group.

sequence CTGCTGCCA between nucleotides −276 and −284, which is very similar to RB1 (CTGCTTGCCA). In *P. aeruginosa*, the sequence CGTTGGN$_{17}$TATAAT is recognized by the RNAP containing the housekeeping σ^D (38); we found the sequence ATTTGCN$_{16}$TATAAT between nucleotides −10 and −35. A DNA sequence similar to that of the promoters recognized by RNAP containing the σ^{54} subunit (33) is present between nucleotides −288 and −304; this sequence does not seem to be a functional promoter by primer extension analysis.

Identification of *algD* mRNA start sites. The mRNA start site for the *algD* gene was determined by primer extension. Two start sites were found in strain ATCC 9046, corresponding to nucleotides −114 and +1, respectively (Fig. 1); thus, the functional promoters which are predominantly used in this strain when grown fixing nitrogen in BS medium include a promoter which is similar to those transcribed by the RNAP-containing σ^E subunit (P2), which is located between nucleotides −124 and −149, with the sequence GTAAN$_{20}$C (Fig. 1). The other promoter (P1) is located at nucleotides −10 to −35 and is a σ^D type of promoter (Fig. 1). No primer extension products were observed in strain UW136.

Construction and characterization of *algD* mutants. To determine whether the putative *algD* gene which had been sequenced was functional in *A. vinelandii*, it was disrupted in strain ATCC 9046. The *algD* disruption of the mutant strain RSD1 (Table 1) was confirmed by Southern blotting (Fig. 3), and it was found to lack alginate production (Table 2). In plates with a high RSD1 cell density, mucoid colonies are apparent (data not shown); this reversion is thought to appear because of genetic recombination between the truncated *algD* genes.

Alginate has been proposed to be essential for encystment, since the nonmucoid strain UW is unable to form mature cysts (36); however, the nature of the mutation leading to nonmucoidy in strain UW was not known. To determine whether alginate was needed for encystment, the formation of desiccation-resistant cells of ATCC 9046 and RSD1 cultures was measured. Strain ATCC 9046 has a frequency of cyst formation of 10%, while the mutant RSD1 has a frequency of less than 10^{-6}. This result supports the assumption that alginate is essential for cyst formation.

Correlation between *algD* transcription and alginate production in different *A. vinelandii* strains. To determine the correlation between *algD* transcription and the amount of alginate produced by different *A. vinelandii* strains, an *algD-lacZ*

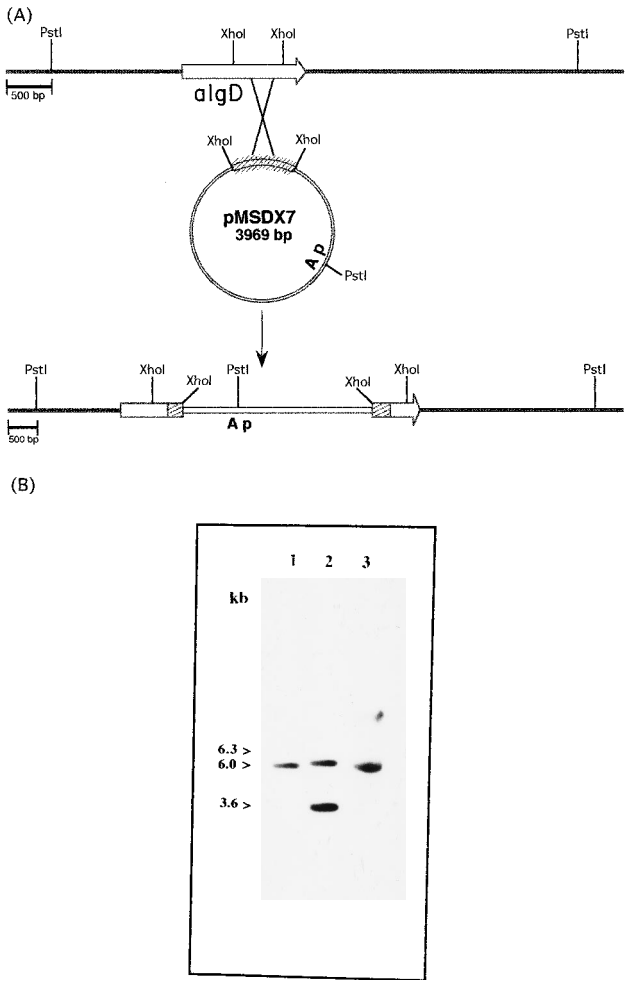

FIG. 3. Insertional inactivation of the *algD* gene in *A. vinelandii* ATCC 9046. (A) Schematic representation of events leading to *algD* inactivation with plasmid pMSDX7, which contains an internal *algD* fragment; (B) Southern blot analysis of *algD* inactivation by insertion of plasmid pMSDX7 in strain ATCC 9046, giving rise to mutant RSD1. DNAs were digested with *Pst*I endonuclease and were hybridized with plasmid pMSDX7. Lanes correspond to ATCC 9046 (*algD*$^+$) (lane 1), RSD1 (*algD*::pMSDX7) (lane 2), and plasmid pMSD675 (*algD*$^+$) (lane 3).

TABLE 2. Alginate production and *algD* expression on BS medium in different *A. vinelandii* strains

Strain	Characteristic(s)	Alginate production ± SD (mg/mg of protein)[a]	β-Galactosidase (U) ± SD
ATCC 9046	Highly mucoid	4.47 ± 0.47	
RSD1	ATCC 9046 *algD* mutant	>0.3	
WI12	ATCC 9046 *algD-lacZ*	1.13 ± 0.18	831 ± 13
AEIV	Mucoid	1.74 ± 0.2	
A2	AEIV *algD-lacZ*	0.53 ± 0.13	285 ± 39
UW136	Nonmucoid	0.73 ± 0.21	
U5	UW136 *algD-lacZ*	0.68 ± 0.31	113 ± 8

[a] These values were determined from liquid cultures incubated at 30°C and at 250 rpm for 48 h.

transcriptional fusion was constructed on the chromosomes of three strains with different levels of mucoidy. The substitution of the original *algD* gene with the *algD-lacZ* fusion on the chromosomes of these strains was confirmed by Southern blot analysis (Fig. 4).

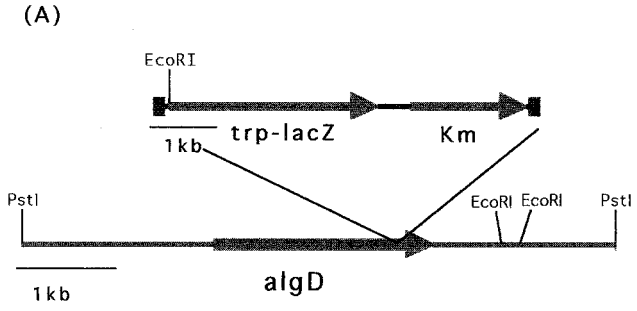

FIG. 4. Construction of *algD-lacZ* transcriptional fusion in *A. vinelandii* strains. (A) Schematic representation of the *algD*-mini-Tn*5lacZ1* insertion located in plasmid pCTD273; (B) Southern blot hybridization of total genomic DNA digested with *Pst*I endonuclease with pCTD273 as a probe. Lanes: 1, ATCC 9046; 2, WI12; 3, AEIV; 4, A2; 5, UW136; and 6, U5.

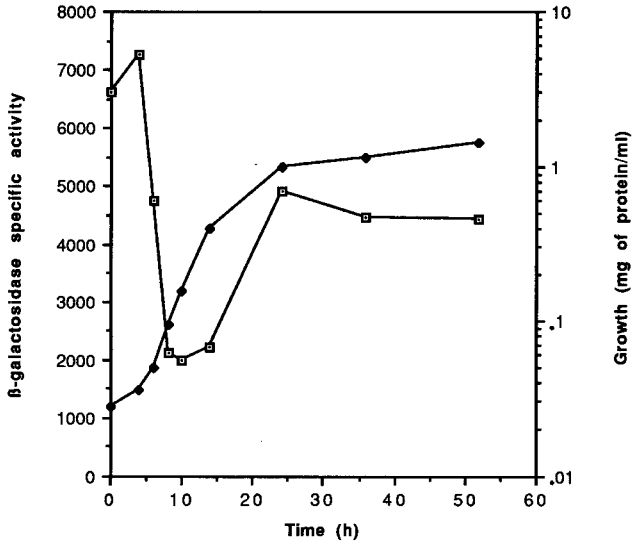

FIG. 5. Expression of the *algD* gene of strain WI12 during the growth cycle. *algD* transcription was measured as β-galactosidase activity units, and the growth of the bacteria was determined by the increment in protein concentrations.

None of the derivatives carrying the *algD-lacZ* fusion on the chromosome is completely impaired in alginate production. Strain WI12, for example, is still able to produce 25% as much alginate as the wild-type strain (Table 2).

As shown in Table 2, there is a strict correlation between *algD* transcription, as measured by the β-galactosidase activities and alginate production of the original strains studied. Strain WI12, which was derived from strain ATCC 9046 (the most mucoid strain), had the highest β-galactosidase activity, while strain U5, which is derived from the nonmucoid strain UW136, presented the lowest values of this enzymatic activity. Strain A2 had an intermediate β-galactosidase activity value and is derived from strain AEIV, which produces intermediate alginate levels.

Regulation of *algD* transcription and alginate production by different environmental stimuli. The kinetics of *algD* transcription during the growth of strain WI12 was determined by measuring β-galactosidase activity (Fig. 5). During the exponential phase of growth, *algD* expression is reduced; it is induced during the late logarithmic phase and is maintained at high levels during the stationary phase.

To simultaneously measure alginate production and β-galactosidase activities under different environmental conditions, we transferred plasmid pMSD675 to the three strains containing the *algD-lacZ* transcriptional fusion.

The environmental stimuli that we studied were medium osmolarity and nitrogen source. Neither alginate production nor *algD* transcription is increased in high-osmolarity medium in any strain; on the contrary, a slight decrease of both parameters is apparent with the higher sodium chloride concentration used (Table 3). Levels of alginate production and *algD* transcription were both very low in strain U5/pMSD675 and were not affected by any of the environmental stimuli studied (Table 3). In the case of strain WI12/pMSD675, we found that alginate production and β-galactosidase activity were always very high, independently of the culture conditions (Table 3). The *algD* transcription level was increased in the A2/pMSD675 background when the strain was grown with ammonium as the nitrogen source while alginate production remained unaltered (Table 3). Both β-galactosidase activity and alginate produc-

TABLE 3. Alginate production and *algD* expression on BS medium with different supplements in three *A. vinelandii* strains

Strain and supplement	Amt of alginate ± SD (mg/mg of protein)	β-Galactosidase activity (U) ± SD
WI12/pMSD675	5.37 ± 0.3	5,859 ± 306
—[a]	3.76 ± 0.24	6,180 ± 303
NaCl (50 mM)[a]	3.59 ± 0.29	6,094 ± 385
NaCl (100 mM)[a]	2.76 ± 0.29	3,318 ± 105
NO_3[b]	4.92 ± 0.04	5,177 ± 139
NH_4[b]	4.82 ± 0.39	4,805 ± 237
A2/pMSD675	2.83 ± 0.13	1,550 ± 120
NaCl (50 mM)[a]	ND[c]	1,718 ± 80
NaCl (100 mM)[a]	ND	1,451 ± 207
NO_3[b]	0.91 ± 0.16	559 ± 54
NH_4[b]	2.46 ± 0.15	2,668 ± 162
U5/pMSD675	0.43 ± 0.006	69 ± 5
NaCl (50 mM)[a]	ND	89 ± 3
NaCl (100 mM)[a]	ND	91 ± 2
NO_3[b]	0.50 ± 0.002	101 ± 0.55
NH_4[b]	0.43 ± 0.35	77 ± 1.53

[a] Measurement done at 48 h of growth on BS medium.
[b] Measurements done at 72 h of growth on BS medium.
[c] ND, not determined.

tion were considerably reduced when strain A2/pMSD675 was grown with nitrate as the nitrogen source (Table 3).

DISCUSSION

Here, we report the cloning and characterization of the *A. vinelandii* gene coding for the GDP-mannose dehydrogenase (*algD*), which is a key enzyme in alginate biosynthesis. We found that it has a high degree of homology with the corresponding *P. aeruginosa algD* gene (8). Primer extension analysis showed the existence of a functional promoter which contains all of the invariable nucleotides of σ^E promoters (18).

In *E. coli* and *P. aeruginosa*, σ^E is involved in the response to environmental stress (18, 29). In *P. aeruginosa*, the σ^E-like protein AlgU has been shown to be involved in the transcription of *algD*; this transcription is dependent on the positive regulator AlgR, which belongs to the two-component class of transcription regulators (9). *A. vinelandii* has been shown to possess DNA sequences homologous to those of *P. aeruginosa algR* (13). Our finding of an *algD* upstream sequence similar to the AlgR binding sequence strongly suggests that the transcription of this *A. vinelandii* gene is regulated by AlgR via activation of the AlgU recognized promoter.

The second *algD* mRNA start site found in strain ATCC 9046 corresponds to a promoter transcribed by the RNAP containing a σ^D subunit (38), the form of the enzyme which transcribes the housekeeping genes. The high level of homology of the σ^D *algD* promoter with the consensus sequence of this type of promoter in *P. aeruginosa* suggests that its transcription is constitutive; however, the consensus sequence for the σ^D homolog in *A. vinelandii* has not been reported yet.

The levels of transcription of *algD* in strains WI12 and A2 are increased by more than fourfold upon the introduction of plasmid pMSD675 (Table 2). This result suggests either that a positive transcription activator is encoded in this plasmid or that a negative regulator is titrated out by *cis*-acting sites contained in plasmid pMSD675.

Transcription of the *algD* gene on the three strains studied showed a different response to the environmental factors studied. High osmolarity was reported to be the environmental condition triggering alginate production by *P. aeruginosa*; however, it was later reported that in different mucoid isolates presenting different *muc* mutations, *algD* expression was induced by different environmental stimuli (2, 10), a situation which is similar to that reported here for *A. vinelandii*. Two of the studied strains have a regulatory mutation, as shown in the accompanying paper (28). Strain UW136 contains a truncated *algU* gene, while strain ATCC 9046 contains a mutation designated *muc-1*, which produces a highly mucoid phenotype. However, it is shown in the present paper that strain ATCC 9046 does not have constitutive *algD* expression, since its transcription is regulated during the different growth phases (Fig. 5).

We have found that a mutation in the unique *A. vinelandii rpoN* gene in strain UW136 has no significant effect on alginate production (data not shown). However, the nitrogen source regulates *algD* expression in the genetic background of strain AEIV (Table 3), which is presumably a wild-type strain. To obtain more-conclusive evidence for the presence of a σ^{54} promoter in strain AEIV, alginate production and *algD* transcription in an *rpoN* mutant derived from it are being determined with different nitrogen sources to study its regulation.

We have shown here that strain RSD1, which contains a disrupted *algD* gene, was unable to produce alginate while a strain, WI12, with a different mutation in the same gene caused by a mini-Tn*5lacZ1* insertion showed only a reduction in the production of this polysaccharide; both mutants were complemented for alginate production by plasmid pMSD675 (Tables 2 and 3 and data not shown). The different phenotypes of these mutants might be due to the fact that the mini-Tn*5lacZ1* insertion in strain WI12 is located at the 3′ end of the gene, rendering a GDP-mannose dehydrogenase with residual activity.

One of the aims of our research is to study the role of alginate biosynthesis in the *A. vinelandii* differentiation process leading to cyst formation. In this respect, we have shown that a mutant impaired in alginate biosynthesis is unable to form desiccation-resistant cells. The precise role of alginate in cyst formation in different strains and in the *alg* mutants derived from them is being analyzed at the ultrastructural level.

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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. **Berry, A., J. D. DeVault, and A. M. Chakrabarty.** 1989. High osmolarity is a signal for enhanced *algD* transcription in mucoid and nonmucoid *Pseudomonas aeruginosa* strains. J. Bacteriol. **171:**2312–2317.
3. **Blumenkrantz, N., and G. Asboe-Hansen.** 1973. New method for quantitative determination of uronic acids. Anal. Biochem. **54:**484–489.
4. **Chakrabarty, A. M.** 1991. Molecular genetics and environmental regulation of alginate biosynthesis. Appl. Phycol. **8:**1–6.
5. **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.
6. **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.
7. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. Methods Enzymol. **235:**386–405.
8. **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.

9. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. *Pseudomonas aeruginosa* infection in cystic fibrosis: nucleotide sequence and transcriptional regulation of the *algD* gene. Nucleic Acids Res. **15:**4567–4581.
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. **Fialho, A. M., N. A. Zielinski, W. F. Fett, A. M. Chakrabarty, and A. Berry.** 1990. Distribution of alginate gene sequences in the *Pseudomonas* rRNA homology group I-*Azomonas-Azotobacter* lineage of superfamily B procaryotes. Appl. Environ. Microbiol. **56:**436–443.
14. **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.
15. **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.
16. **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.
17. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli*. J. Mol. Biol. **166:**557–580.
18. **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 (σ^E). Proc. Natl. Acad. Sci. USA **92:**7941–7945.
19. **Jarman, T. R., L. Deavin, S. Slocombe, and R. C. Righelato.** 1978. Investigation of the effect of environmental conditions on the rate of exopolysaccharide synthesis in *Azotobacter vinelandii*. J. Gen. Microbiol. **107:**59–64.
20. **Kennedy, C., R. Gamal, R. Humphrey, J. Ramos, K. Brigle, and D. Dean.** 1986. The *nifH*, *nifM* and *nifN* genes of *Azotobacter vinelandii*: characterization by Tn*5* mutagenesis and isolation from pLAFR1 gene banks. Mol. Gen. Genet. **205:**318–325.
21. **Kennedy, C., and A. Toukdarian.** 1987. Genetics of azotobacters: applications to nitrogen fixation and related aspects of metabolism. Annu. Rev. Microbiol. **41:**227–258.
22. **Knutson, C. A., and A. Jeanes.** 1968. A new modification of the carbazole reaction: application to heteropolysaccharides. Anal. Biochem. **24:**470–481.
23. **Kokotek, W., and W. Lotz.** 1991. Construction of a mobilizable cloning vector for site-directed mutagenesis of Gram-negative bacteria: application to *Rhizobium leguminosarum*. Gene **98:**7–13.
24. **Maldonado, R., J. Jiménez, and J. Casadesus.** 1994. Changes in ploidy during the *Azotobacter vinelandii* growth cycle. J. Bacteriol. **176:**3911–3919.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Manna, A. C., and H. K. Das.** 1993. Determination of the size of the *Azotobacter vinelandii* chromosome. Mol. Gen. Genet. **241:**719–722.
27. **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 σ^E and stress response. J. Bacteriol. **176:**6688–6696.
28. **Martínez-Salazar, J. M., S. Moreno, R. Nájera, J. C. Boucher, G. Espín, G. Soberón-Chávez, and V. Deretic.** 1996. 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. J. Bacteriol. **178:**1800–1808.
29. **May, T. B., and A. M. Chakrabarty.** 1994. *Pseudomonas aeruginosa*: genes and enzymes of alginate synthesis. Trends Microbiol. **2:**151–157.
30. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431–435. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Mohr, C. D., N. S. Hibler, and V. Deretic.** 1991. AlgR, a response regulator controlling mucoidy in *Pseudomonas aeruginosa*, binds to the FUS sites of the *algD* promoter located unusually far upstream from the RNA start site. J. Bacteriol. **173:**5136–5143.
32. **Mohr, C. D., D. W. Martin, W. N. Konyecsni, J. R. W. Govan, S. Lory, and V. Deretic.** 1990. Role of the far-upstream sites of the *algD* promoter and the *algR* and *rpoN* genes in environmental modulation of mucoidy in *Pseudomonas aeruginosa*. J. Bacteriol. **172:**6576–6580.
33. **Morett, E., and M. Buck.** 1989. In vivo studies on the interaction of RNA polymerase-σ^{54} with the *Klebsiella pneumoniae* and *Rhizobium meliloti nifH* promoters: the role of NifA in the formation of an open promoter complex. J. Mol. Biol. **210:**65–77.
34. **Nagpal, P., M. A. Reddy, and H. K. Das.** 1989. Multiple chromosomes of *Azotobacter vinelandii*. J. Bacteriol. **171:**3133–3138.
35. **Page, W., and H. L. Sadoff.** 1975. Relationship between calcium and uronic acids in the encystment of *Azotobacter vinelandii*. J. Bacteriol. **122:**145–151.
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. **Ronald, S., M. A. Farinha, B. J. Allan, and A. M. Kropinski.** 1992. Cloning and physical mapping of transcriptional regulatory (sigma) factors from *Pseudomonas aeruginosa*, p. 249–257. *In* E. Galli, S. Silver, and B. Withold (ed.), *Pseudomonas*: molecular biology and biotechnology. American Society for Microbiology, Washington, D.C.
39. **Schurr, M. J., D. W. Martin, M. H. Mudd, and V. Deretic.** 1994. Gene cluster controlling conversion to alginate-overproducing phenotype in *Pseudomonas aeruginosa*: a functional analysis in a heterologous host and role in the instability of mucoidy. J. Bacteriol. **176:**3375–3382.
40. **Schurr, M. J., D. W. Martin, M. H. Mudd, N. S. Hibler, 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.
41. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
42. **Sutherland, I. W.** 1985. Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharides. Annu. Rev. Microbiol. **39:**243–270.
43. **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.
44. **Wyss, O., G. M. Neumann, and M. D. Scolofsky.** 1961. Development and germination of the *Azotobacter* cyst. J. Biophys. Biochem. Cytol. **10:**555–565.