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Role of Alternative σ Factor AlgU in Encystment of *Azotobacter vinelandii*

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Alginate is essential for encystment in *Azotobacter vinelandii*. Transcription of the *algD* gene, which codes for GDP-mannose dehydrogenase, a key enzyme in the alginate biosynthetic pathway, is initiated at two promoters, one of which, p2, has σ^E consensus sequences. AlgU is the *A. vinelandii* alternative σ^E factor. In this study, we constructed an *algU* mutant (SMU88) which, as expected, is impaired in alginate production, encystment, and transcription of the *algD* gene from the p2 promoter. Plasmid pJMSAT1, carrying the *A. vinelandii algU* gene, restored alginate production and encystment to SMU88 and to strain UW136, a naturally occurring *algU* mutant. Plasmid pSMU865, carrying the *A. vinelandii mucABCD* genes coding for negative regulators of AlgU activity and previously shown to diminish alginate production in the wild-type strain, ATCC 9046, was shown here to impair encystment and transcription of the *algD* gene from the p2 *algU*-dependent promoter. Since nonencysting strain ATCC 9046/pSMU865 produced more alginate than some encysting strains, such as UW136/pJMSAT1, we propose an AlgU role in encystment, independent of the structural role that alginate plays in mature cysts.

Encystment in *Azotobacter vinelandii* has been studied at the morphological and biochemical levels (9, 10, 22, 27). This differentiation process is induced by growth on *n*-butanol as the sole carbon source (12); mature cysts develop in 3 to 5 days and are characterized by a central body surrounded by an intine and a thick, laminated exine. Alginate is essential for encystment since it is a component of intine and exine layers (1, 3). The alginate biosynthetic pathways of *A. vinelandii* and *Pseudomonas aeruginosa* are very similar (21). The molecular genetics of alginate biosynthesis have been widely studied in *P. aeruginosa* (for reviews, see references 5 and 17), where the genes encoding the alginate biosynthetic enzymes are organized in an operon starting with *algD* (4), encoding a GDP-mannose dehydrogenase, which converts GDP-mannose to GDP-mannuronic acid, the substrate for alginate polymerization. The *algU mucABCD* cluster controls alginate production. AlgU (also known as AlgT) is a σ^E homolog (8); it is required for transcription of *algD* and its own gene, *algU* (15, 25). The *mucA* and *mucB* genes code for negative regulators of AlgU activity; MucA interacts with AlgU as an anti-sigma factor, inhibiting its activity (6, 24, 25, 28).

The *A. vinelandii* alginate biosynthetic gene cluster is organized in at least three operons, one of which includes the *algD* gene (3, 13, 18). In the highly mucoid strain *A. vinelandii* ATCC 9046, *algD* is transcribed from two promoters: a putative AlgU dependent promoter, p2, and a promoter (p1) showing consensus sequences for the vegetative sigma factor σ^D (3). We identified and sequenced the *algU* and *mucABCD* genes of *A. vinelandii* and presented evidence showing that they control alginate biosynthesis in a manner similar to that of the *P. aeruginosa* homologs (16).

An *A. vinelandii* strain unable to produce alginate due to a mutation in the *algD* gene is unable to encyst (3). The naturally occurring *A. vinelandii* strain UW136 does not produce alginate, due to a mutation in the *algU* gene, which codes for the AlgU sigma factor (16), and is also unable to encyst (20). The question of whether *algU* mutations affect encystment exclu-

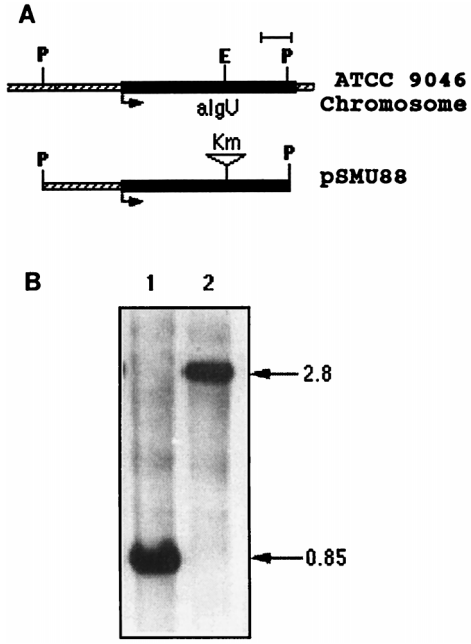

FIG. 1. Construction of strain SMU88. (A) Schematic representation of the *A. vinelandii algU* region and the *algU*::Km mutation in plasmid pSMU88. Bar, 100 bp. (B) Southern blot hybridization of total genomic DNA digested with *Pst*I endonuclease, with pSMU85 as a probe. Lanes: 1, ATCC 9046; 2, SMU88. Molecular sizes (in kilobases) are indicated on the right.

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TABLE 1. Alginate production and encystment[a]

Strain	Alginate (mg/mg of protein)	Resistance to desiccation (%)
ATCC 9046	2.5 ± 0.50	7.2 ± 1.5
ATCC 9046/pSMU865	0.46 ± 0.04	<0.0001
SMU88	0.003 ± 0.001	<0.0001
SMU88::pJMSAT1	2.74 ± 0.7	7.1 ± 2.5
SMU88/pDMUM13	0.170 ± 0.04	1.9 ± 0.5
UW136	0.004 ± 0.002	<0.0001
UW136::pJMSAT1	0.19 ± 0.02	8.8 ± 2.5
UW136/pDMUM13	0.16 ± 0.03	4.0 ± 1.0

[a] Alginate production was determined in cells induced for encystment. Cells grown during 5 days on BS plates with 0.2% *n*-butanol as the sole carbon source were used to measure alginate production, as described previously (19). Encystment was determined as previously described (3). All values are means of three determinations (± standard deviations, where appropriate).

sively via its effect on alginate biosynthesis or whether AlgU activity is also required for expression of other genes involved in cyst formation was raised. In the present work we present evidence suggesting that AlgU is involved in the formation of mature cysts, independently of its role in alginate production.

Construction and characterization of an ATCC 9046 *algU* mutant. To study the role of AlgU in encystment, we constructed an ATCC 9046 derivative carrying an *algU*::Km null mutation. A *Pst*I DNA fragment of 828 nucleotides corresponding to *algU* from *A. vinelandii* ATCC 9046 (Fig. 1) was cloned into plasmid pBluescript II KS to give pSMU85. A 2-kb *Sma*I fragment containing a kanamycin resistance gene from plasmid pHP45Ω-Km (7) was inserted into the unique *Eco*RV site present within the 828-bp fragment of pMSU85 to create an *algU*::Km mutation between the codons for amino acid residues 115 and 116 of AlgU. The resultant plasmid, pSMU88, which is unable to replicate in *A. vinelandii*, was introduced into strain ATCC 9046 by transformation. One of the kanamycin-resistant transformants, strain SMU88, was chosen for further analysis. The replacement of the intact *algU* gene with the *algU*::Km mutation on the chromosome of the SMU88 mutant was confirmed by Southern blotting (Fig. 1). The *algU* mutation impaired alginate production and encystment (Table 1). To complement the *algU* mutation, a 1.6-kb DNA fragment with the *algU-mucA* region from ATCC 9046, obtained by PCR, was cloned into plasmid pCRII, which confers ampicillin resistance (2). The resultant plasmid, pJMSAT1, which is unable to replicate in *A. vinelandii*, was transformed into *algU* mutant strain SMU88. A transformant, SMU88::pJMSAT1, carrying plasmid pJMSAT1 integrated into the chromosome, was selected as a mucoid colony resistant to carbenicillin. Integration of the plasmid was confirmed by Southern blot analysis (data not shown). Strain SMU88::pJMSAT1 was able to encyst (Table 1). We also show that plasmid pDMUM13 carrying the *P. aeruginosa algU* gene (14), previously shown to restore alginate production to strain UW136 (16), also restored encystment and alginate production to strains SMU88 and UW136 (Table 1). Although the amounts of alginate synthesized by strains SMU88, UW136 complemented with plasmid pDMUM13, and UW136::pJMSAT1 were significantly lower than that of ATCC 9046, they were sufficient for encystment (Table 1).

Effects of plasmid pSMU865 and the *algU*::Km mutation on transcription from the *algD* p1 and p2 promoters. Plasmid pSMU865, carrying the *A. vinelandii mucABCD* genes, was previously shown to reduce alginate production in strain ATCC 9046 (16). We propose that the effect of pSMU865 on alginate production in ATCC 9046 is due to the negative effect of the *mucA* and *mucB* gene products on AlgU activity and therefore on transcription from $algDp_2$. Thus, transcription of the *algD* gene in strains ATCC 9046/pSMU865 and SMU88 should initiate predominantly from p1, the AlgU-independent promoter. Primer extension experiments were performed on RNA isolated from strains SMU88 and ATCC 9046 in the presence and absence of plasmid pSMU865 and grown in liquid BS medium (11) for 48 h at 30°C. Reactions were performed with a primer extension system (Boehringer), as instructed by the manufacturer. Oligonucleotide primers used were labeled with [γ-^{32}P]dATP (Amersham) at the 5′ end by

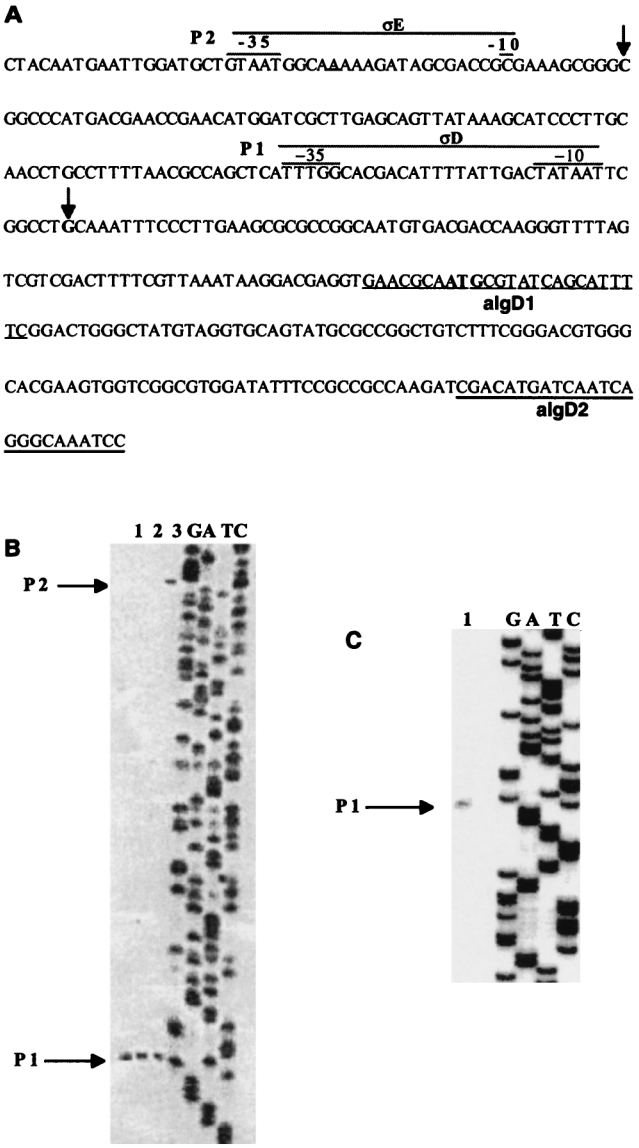

FIG. 2. Primer extension analysis of *algD* transcription in strains SMU88 and ATCC 9046, with and without plasmid pSMU865. (A) DNA sequence of the 5′ end of *algD*. The arrows indicate the start sites of *algD* transcription, the p1 and p2 promoters are indicated (overbar), and the complementary sequences where oligonucleotides *algD*1 and *algD*2 (used for primer extension analysis) were generated are underlined. The *algD* ATG initiation codon is shown in boldface type. (B and C) Primer extension of the *algD* gene with oligonucleotide *algD*1 in strains SMU88 (lane 1) and ATCC 9046 with (lane 2) and without (lane 3) plasmid pSMU865 (B) and with oligonucleotide *algD*2 in strain SMU88 (C). The *algD* sequence ladders (GATC) were produced with the oligonucleotides used for primer extension.

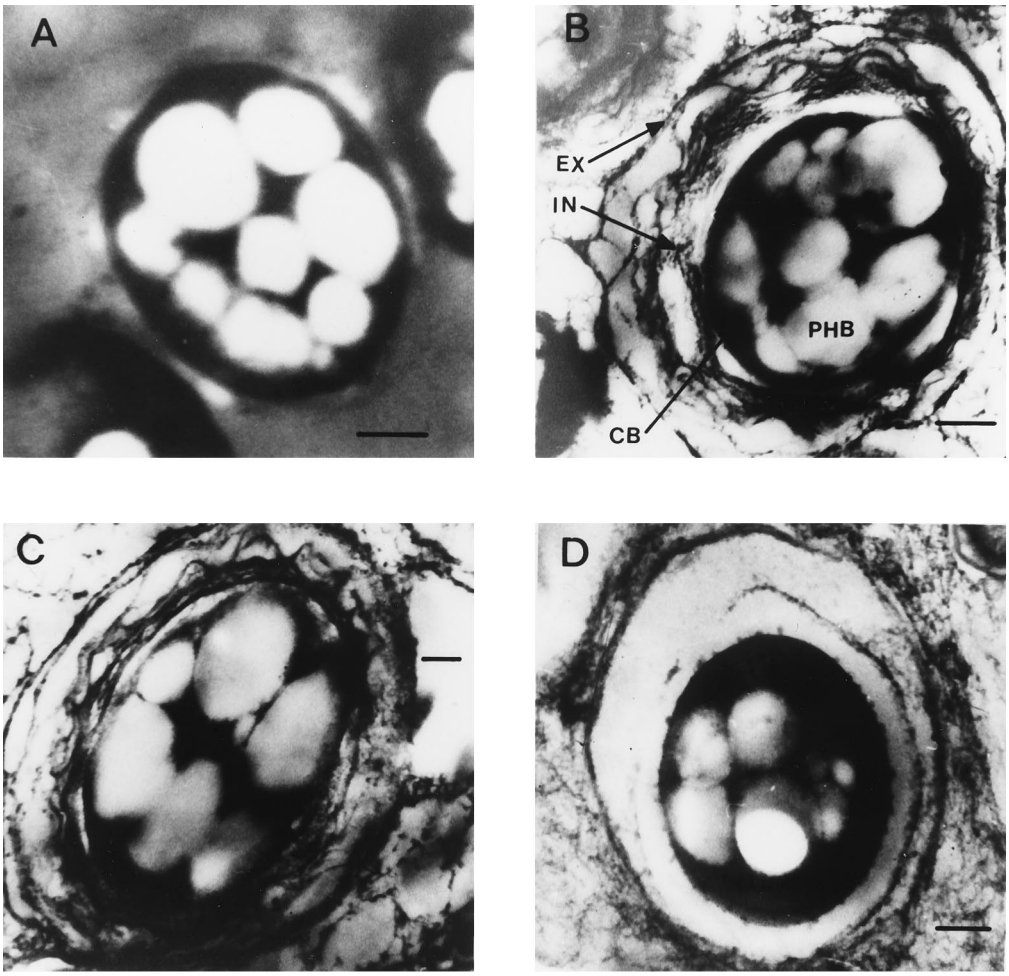

FIG. 3. Electron micrographs were produced as described previously (19). Thin sections of *A. vinelandii* cysts formed by strains SMU88 (A), SMU88/pDMUM13 (B), ATCC 9046 (C), and ATCC 9046/pSMU865 (D) are shown. Abbreviations: EX, exine; IN, intine; CB, central body; PHB, poly-β-hydroxybutyrate. Bars, 0.4 μm.

using polynucleotide kinase and were hybridized to 50 μg of total RNA. After extension with reverse transcriptase, cDNA products were examined by electrophoresis in an 8% polyacrylamide gel. To map transcriptional start points, sequencing reactions were performed on pMSD27 (16) DNA by the dideoxy chain termination method (23) with [γ-^{32}P]dATP and a sequencing kit with the same primers as used for the primer extension reactions.

As expected, primer extension products corresponding to p1 and p2 transcripts were observed with ATCC 9046 RNA, but only the primer extension products corresponding to p1 transcripts were observed with RNA from strains SMU88 and ATCC 9046/pSMU865, confirming that p2 is an AlgU-dependent promoter (Fig. 2).

In *P. aeruginosa*, *algU* mutants are unable to produce alginate, since transcription of the biosynthetic operon starting with *algD*, as well as some regulatory genes such as *algR*, is dependent on the presence of an active AlgU sigma factor (15, 26). Similarly, *A. vinelandii algU* mutants UW136 and SMU88 are also unable to produce alginate; in contrast to *P. aeruginosa*, however, in *A. vinelandii* transcription of all other known alginate biosynthetic genes, such as *algA* and *alg8*, is not dependent on AlgU (13, 18). As shown here, AlgU is absolutely required for transcription from $algDp_2$, but *algD* is still transcribed from $algDp_1$ in strain ATCC 9046. Transcription from $algDp_1$ was also detected in strain SMU88, with an oligonucleotide corresponding to the nucleotides coding for amino acids 37 to 44 of AlgD (Fig. 2), indicating that transcription from p1 extends into the *algD* structural gene. Thus, the fact that an *algU* mutation abolished alginate production in this strain indicates that in *A. vinelandii*, transcription of other unidentified regulatory or biosynthetic alginate genes depends on AlgU.

Effect of plasmid pSMU865 on encystment. We studied encystment in strain ATCC 9046/pSMU865, which produces alginate even though its AlgU activity is not sufficient to initiate transcription from $algDp_2$. Table 1 shows that this strain was unable to form desiccation-resistant cysts, despite the fact that under encysting conditions it produced more alginate than the encysting strains UW136::pJMSAT1, SMU88/pDMUM13, and UW136/pDMUM13. These data strongly suggest that AlgU plays an additional role in the expression of genes involved in cyst formation. The observation that an *algU* mutation, but not plasmid pSMU865, abrogated alginate production in strain ATCC 9046 leads us to hypothesize that in strain ATCC 9046/pSMU865, a low level of AlgU activity allows some transcription of the unidentified alginate genes mentioned above but not of *algD* (from p2) and encystment genes.

The putative AlgU requirement for transcription of genes involved in encystment may allow the identification and characterization of such genes.

Ultrastructure analysis of cyst formation. Electron microscopic examination of the cyst structures formed by the mutant strains (Fig. 3) showed that strain SMU88 lacks the intine and exine layers of mature cysts such as those produced by the wild-type strain, ATCC 9046. SMU88/pDMUM13 cysts were similar to those produced by wild-type strain ATCC 9046, consisting of the compacted cell (central body containing poly-β-hydroxybutyrate granules) surrounded by the intine capsule and the exine outer shell. The cyst structures formed by strain ATCC 9046/pSMU865 (Fig. 3D) appear to lack the intine layers. An early electron microscopy study of the development of *A. vinelandii* cysts (27) revealed that the exine appears in 36 to 48 h, after which the exine thickens and the intine is formed between the exine and the central body; thus, at 36 h the intine appears to be nothing but an empty area. This seems to be the stage at which encystment development is blocked in the absence of AlgU activity.

This study contributes to our understanding of the *A. vinelandii* differentiation process leading to cyst formation at the molecular level and is the first example of the involvement of an alternative sigma factor in cellular differentiation of a gram-negative bacterium.

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