

# Inactivation of the *ampDE* Operon Increases Transcription of *algD* and Affects Morphology and Encystment of *Azotobacter vinelandii*

CINTHIA NÚÑEZ, SOLEDAD MORENO, LUIS CÁRDENAS, GLORIA SOBERÓN-CHÁVEZ,  
AND GUADALUPE ESPÍN\*

Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México,  
Cuernavaca Morelos 62250, México

Received 7 March 2000/Accepted 1 June 2000

**Transcription of *algD*, encoding GDP-mannose dehydrogenase, the key enzyme in the alginate biosynthetic pathway, is highly regulated in *Azotobacter vinelandii*. We describe here the characterization of a Tn5 insertion mutant (AC28) which shows a higher level of expression of an *algD::lacZ* fusion. AC28 cells were morphologically abnormal and unable to encyst. The cloning and nucleotide sequencing of the Tn5-disrupted locus in AC28 revealed an operon homologous to the *Escherichia coli ampDE* operon. Tn5 was located within the *ampD* gene, encoding a cytosolic *N*-acetyl-anhydromuramyl-L-alanine amidase that participates in the intracellular recycling of peptidoglycan fragments. The *ampE* gene encodes a transmembrane protein, but the function of the protein is not known. We constructed strains carrying *ampD* or *ampE* mutations and one with an *ampDE* deletion. The strain with a deletion of the *ampDE* operon showed a phenotype similar to that of mutant AC28. The present work demonstrates that both alginate production and bacterial encystment are greatly influenced by the bacterial ability to recycle its cell wall.**

*Azotobacter vinelandii* is a soil bacterium that undergoes a process of cellular differentiation to form metabolically dormant cysts resistant to desiccation (for a review, see reference 38). Wild-type strains of *A. vinelandii* produce the extracellular polysaccharide alginate, a linear copolymer of D-mannuronic acid and its C-5 epimer L-guluronic acid. Alginate is essential for the encystment process, since it is a component of the intine and exine layers of the cysts (31) and since nonmucoid strains fail to form cysts (5, 26, 29).

The pathway for alginate biosynthesis in *A. vinelandii* has been elucidated (36); fructose-6-P is converted by four enzymatic reactions to GDP-mannuronic acid, which is the substrate of the polymerase. The resultant polymannuronic acid is secreted and modified by an *O*-acetylase and several extracellular C-5 epimerases to give the final product, alginate (9, 36, 37).

The *algD* gene codes for GDP-mannose dehydrogenase, which converts GDP-mannose, a metabolite used for the synthesis of different saccharides, to GDP-mannuronic acid. *algD* is located in a biosynthetic gene cluster which contains most of the genes that code for the enzymes involved in the synthesis and modification of alginate (5, 23, 26, 27, 37, 42). A correlation between alginate production and *algD* transcription was found for three *A. vinelandii* strains producing different alginate levels (5).

In *Pseudomonas aeruginosa*, the  $\sigma^E$  factor AlgU (also known as AlgT) controls transcription of the alginate biosynthetic operon and is negatively regulated by MucA, an inner membrane protein that has been shown to act as an antisigma factor, and MucB, a periplasmic protein proposed to sense denatured proteins in the periplasm (45). In *A. vinelandii* the

*algUmucABCD* genes have been characterized and have been shown to regulate alginate production (25, 29). Transcription of the *A. vinelandii algD* gene is initiated from three sites (5, 29, 30); initiation from one of them, p2, requires AlgU (5, 29). Transcription of *algD* is also under the control of the two-component regulatory system GacSA (6).

The bacterial cell wall is composed of a heteropolymer known as murein or peptidoglycan. The peptidoglycan consists of glycan chains of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid that are frequently cross-linked by short peptides to each other (34). *Escherichia coli* and presumably most other gram-negative bacteria degrade up to 50% of their murein per generation; however, most of the liberated murein fragments (1-6-anhydro-*N*-acetylmuramyl [MurNAc]-L-Ala-D-Glu-mA2pm) are transported from the periplasm into the cytoplasm to reutilize the tripeptide L-Ala-D-Glu-A2pm to form new murein (12, 13, 32).

In enterobacteria the *ampD* and *ampE* genes form an operon (15, 22). The *ampE* gene encodes an inner membrane protein with an ATP-binding motif whose function is unknown (15, 22). The *ampD* gene encodes a cytosolic *N*-acetylmuramyl-L-alanine amidase essential for murein tripeptide recycling. AmpD is specific for anhydro-MurNAc-tripeptides (anhydro-MurNAc-L-Ala-D-Glu-mA2pm) (17); thus, it distinguishes the newly synthesized peptidoglycan precursors which lack the anhydrous bond from the peptidoglycan-derived anhydromuropeptides transported back into the cell for recycling (17).

In many gram-negative bacteria, the inducible  $\beta$ -lactamase gene *ampC* is transcriptionally controlled by a regulator encoded by *ampR*, which belongs to the LysR family of transcriptional regulators (21). Mutations in *ampD* result in constitutive expression of AmpC  $\beta$ -lactamase even in the absence of  $\beta$ -lactam antibiotics. AmpR is inhibited in the presence of the main murein precursor UDP-MurNAc-pentapeptide, and this inhibition is reversed by anhydro-MurNAc-tripeptide (18). In *ampD* mutants, the substrate (anhydro-MurNAc-tripeptide) accumulates and acts as an intracellular positive effector for

\* Corresponding author. Mailing address: Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo Postal 510-3, Cuernavaca Morelos 62250, México. Phone: 52-73-114692. Fax: 52-73-172388. E-mail: espin@ibt.unam.mx.

TABLE 1. Bacterial strains and plasmids used in this work

| Strain or plasmid         | Relevant characteristic(s)  | Source or reference |
|---------------------------|---|---------------------|
| <i>A. vinelandii</i>      |   |                     |
| ATCC 9046                 | Highly mucoid   | ATCC                |
| AEIV                      | Mucoid  | Svein Valla         |
| WI12                      | <i>algD::lacZ</i> derivative of ATCC 9046, Km <sup>r</sup>  | 5                   |
| AC28                      | <i>ampD::Tn5</i> derivative of WI12, Tc <sup>r</sup>  | This work           |
| ATD1                      | ATCC 9046 with a nonpolar <i>ampD::Gm</i> mutation  | This work           |
| ATE1                      | ATCC 9046 with an <i>ampE::Sp</i> mutation  | This work           |
| ATDE1                     | ATCC 9046 with a $\Delta$ <i>ampDE::Gm</i> mutation   | This work           |
| AED1                      | AEIV with a nonpolar <i>ampD::Gm</i> mutation   | This work           |
| AEE1                      | AEIV with an <i>ampE::Sp</i> mutation   | This work           |
| AEDE1                     | AEIV with a $\Delta$ <i>ampDE::Gm</i> mutation  | This work           |
| AE28                      | AEIV carrying an <i>ampD::Tn5</i> insertion, Tc <sup>r</sup>  | This work           |
| WID1                      | WI12 with a nonpolar <i>ampD::Gm</i> mutation   | This work           |
| WIE1                      | WI12 with an <i>ampE::Sp</i> mutation   | This work           |
| WIDE1                     | WI12 with a $\Delta$ <i>ampDE::Gm</i> mutation  | This work           |
| <i>E. coli</i>            |   |                     |
| DH5 $\alpha$              | <i>supE44</i> $\Delta$ <i>lacU169</i> <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>                           | 14                  |
| Plasmids                  |   |                     |
| pCP13                     | RK2-derived cosmid vector; Tc <sup>r</sup> Km <sup>r</sup>  | 7                   |
| pBluescript SK+           | Plasmid used for subcloning DNA; Ap <sup>r</sup>  | Stratagene          |
| pKT230                    | Broad-host-range vector; Sm <sup>r</sup> Km <sup>r</sup>  | 2                   |
| pUT-mini-Tn5 <i>luxAB</i> | Suicide vector for mutagenesis with mini-Tn5 <i>luxAB</i> ; able to replicate only in strains expressing $\lambda$ pir protein; Tc <sup>r</sup> | 8                   |
| pRK2013                   | ColE1-tra (RK2); Km <sup>r</sup>  | 11                  |
| pHP45 $\Omega$ -Sp        | Plasmid used to provide an $\Omega$ Sp <sup>r</sup> cassette  | 10                  |
| pBSL141                   | Plasmid used to provide a Gm <sup>r</sup> cassette  | 1                   |
| pK28                      | pBluescript SK+ with a 3.0-kb <i>KpnI</i> fragment containing the site of transposition in AC28; Tc <sup>r</sup>                                | This work           |
| pSM194                    | Cosmid containing 25 kb of <i>A. vinelandii</i> DNA including <i>ampDE</i> operon derived from pCP13; Tc <sup>r</sup>                           | This work           |
| pS194.5                   | pBluescript SK+ with a 2.0-kb <i>SmaI</i> fragment containing <i>ampDE</i> operon   | This work           |
| pS195                     | pS194.5 derivative containing <i>ampD</i> 5' end  | This work           |
| pS196                     | pS194.5 derivative containing <i>ampD</i> gene and <i>ampE</i> 5' end   | This work           |
| pS196.2                   | pS194.5 derivative containing <i>ampE</i> 5' end  | This work           |
| pSD1                      | pS196 derivative containing <i>ampD::Gm</i> mutation  | This work           |
| pSE1                      | pS196.2 derivative containing <i>ampE::Sp</i> mutation  | This work           |
| pSDE1                     | pS194.5 derivative containing $\Delta$ <i>ampDE::Gm</i> mutation  | This work           |
| pKT194.5                  | pKT230 derivative carrying <i>ampDE</i> operon; Sm <sup>r</sup>   | This work           |

*ampC* expression (16). The presence of *ampD*, even in bacteria lacking an inducible *ampC*  $\beta$ -lactamase gene, suggests that cell wall recycling may have a signaling role in other cellular processes (33). In agreement with this suggestion, in *Bacillus subtilis*, transport of wall-derived peptides into the cell has been proposed to have a signaling role in the initiation of sporulation (35).

In this study, we describe the isolation and characterization of a Tn5 insertion mutation which increases transcription of *algD* and, consequently, alginate production in *A. vinelandii*. This was accomplished by isolating a mutant derivative of strain WI12 carrying an *algD::lacZ* fusion that showed a deep blue color on plates containing X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside). The Tn5 insertion was shown to interrupt an operon homologous to *E. coli ampDE*. The results presented here indicate that *A. vinelandii ampD* and *ampE* gene products contribute to normal cell morphology and suggest that cell wall recycling has a signaling role for the control of alginate synthesis and cyst formation in *A. vinelandii*.

#### MATERIALS AND METHODS

**Microbiological procedures.** Bacterial strains and plasmids used are listed in Table 1. Media and growth conditions were as follows. *A. vinelandii* was grown at 30°C in Burk's nitrogen-free salts supplemented with 2% sucrose (BS) (19). *E. coli* DH5 $\alpha$  was grown on Luria-Bertani (LB) medium (28) at 37°C. Antibiotic

concentrations used for *A. vinelandii* and *E. coli*, respectively, were as follows: tetracycline, 20 and 20  $\mu$ g/ml; kanamycin, 2  $\mu$ g/ml (not used for *E. coli*); ampicillin, 100  $\mu$ g/ml (not used for *A. vinelandii*); nalidixic acid, 20 and 10  $\mu$ g/ml; spectinomycin, 100 and 100  $\mu$ g/ml; streptomycin, 2 and 20  $\mu$ g/ml; gentamicin, 1.5 and 10  $\mu$ g/ml.

Triparental and biparental matings were carried out as previously reported (19). *A. vinelandii* transformation was carried out as described by Bali et al. (3).  $\beta$ -Galactosidase activity was measured as reported by Miller (28); 1 U corresponds to 1 nmol of *o*-nitrophenyl- $\beta$ -D-galactoside hydrolyzed per min per  $\mu$ g of protein. Protein was determined by the Lowry method (24). All measurements were done in triplicate.

Alginate production was determined as previously described (26), and all determinations were done in triplicate.

Encystment was determined as previously described (5) by measuring the percentage of cells resistant to desiccation after induction with *n*-butanol.

**Transposon mutagenesis.** Random transposon mutagenesis of WI12 was carried out as described previously with a pUT derivative containing the mini-Tn5-*luxAB* transposon (8). Matings were done on Burk's sucrose (BS)-LB medium plates overnight at 30°C. The cells were resuspended in 10 mM MgSO<sub>4</sub> and plated on BSTcNal plates, and 200 WI12 Tc<sup>r</sup> derivatives were isolated from each mating.

**Cloning of the AC28 transposon insertion.** Southern blot analysis was used to determine suitable restriction fragments on which the transposon-inactivated locus in strain AC28 was to be recovered. We identified a 3-kb *KpnI* fragment which contained the Tc<sup>r</sup> cassette derived from the transposon (data not shown). *KpnI*-digested genomic DNA from AC28 was then size fractionated by agarose gel electrophoresis, purified from the gel, and ligated into pBluescript KS+ (Stratagene), resulting in plasmid pK28. Ligations were transformed into *E. coli* DH5 $\alpha$ , and transformed cells were plated on LB medium containing tetracycline.

**Nucleic acid procedures.** RNA and DNA isolation, cloning, Southern blotting, and nick translation procedures were carried out as described previously (39).

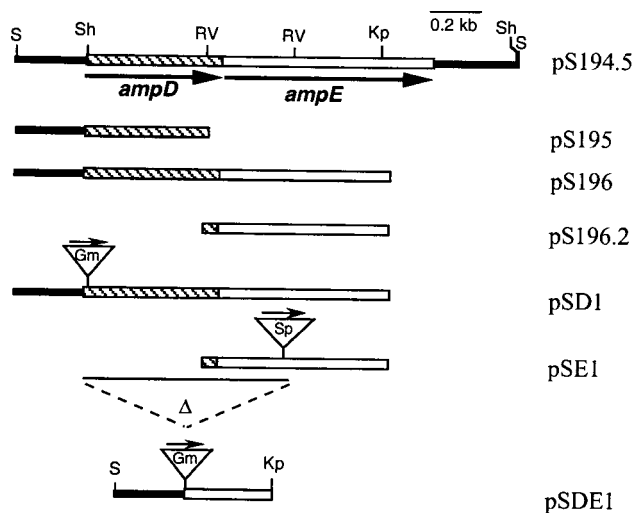


FIG. 1. Physical map of the *A. vinelandii ampDE* region and the plasmids constructed in this study. Arrows, direction of transcription; triangles, antibiotic resistance cassettes. Abbreviations: Kp, *KpnI*; RV, *EcoRV*; S, *SmaI*; Sh, *SphI*.

Plasmids pS194.5, pS195, pS196, and pS196.2 (Fig. 1; Table 1) were used to determine the nucleotide sequence reported in this study. DNA sequencing was done with the *Taq* FS DNA polymerase and fluorescent dideoxy terminators using a cycle sequencing method. The precise site of transposition in AC28 was determined by nucleotide sequencing of pK28 across the transposon insertion junction. Primer extension of *algD* was carried out as previously described (5) using a primer extension kit (Amersham) as instructed by the manufacturer.

**Construction of plasmid pKT194.5 carrying the *A. vinelandii ampDE* operon.** Cosmid clone pSM194, derived from an *A. vinelandii* genomic library, was shown to contain the *ampDE* operon in a 2-kb *SmaI* fragment. This fragment was cloned into the pBluescript KS+ vector to yield plasmid pS194.5 (Fig. 1). The *ampDE* operon was released from this construction as a *Bam*HI-*Hind*III fragment and cloned into the pKT230 vector, previously digested with *Bam*HI and *Hind*III endonucleases, producing plasmid pKT194.5.

**Construction of *ampD*::Gm, *ampE*::Sp, and  $\Delta$ *ampDE*::Gm mutants.** Plasmid pS196 was used to introduce into the unique *Sph*I site of *ampD* a 0.8-kb *SmaI* fragment with a gentamicin resistance (*Gm*<sup>r</sup>) cassette (1), and plasmid pSD1 with the *Gm*<sup>r</sup> cassette ligated in the same orientation as that for the *ampD* transcription was isolated (Fig. 1), generating an *ampD*::Gm nonpolar mutation. Plasmid pS196.2 contains a unique *EcoRV* site within *ampE*. Therefore, in order to construct an *ampE*::Sp mutation, plasmid pS196.2 was cleaved with *EcoRV* and the 2-kb *SmaI* fragment containing an  $\Omega$  spectinomycin resistance cassette (10) was inserted to create pSE1. To generate an *ampDE*::Gm deletion mutant, plasmid pS196 was cleaved with *Sph*I and *EcoRV* (releasing the entire *ampD* gene as well as the 5' end of *ampE*), blunt ended, and ligated to a 0.8-kb *SmaI* fragment containing a gentamicin resistance cassette, resulting in pSDE1. Plasmids pSD1, pSE1, and pSDE1 (Fig. 1), which were unable to replicate in *A. vinelandii*, were used to introduce the *ampD*::Gm, *ampE*::Sp, and  $\Delta$ *ampDE*::Gm mutations into strains ATCC 9046, AEIV, and WI12. Transformants were selected using the corresponding antibiotic and were confirmed by Southern blot analysis to carry the desired mutations (data not shown).

**Microscopic analysis.** *A. vinelandii* cells, grown for 48 h on BS plates, were resuspended, mounted between two coverslips, and visualized under a microscope (Eclipse 330; Nikon) with a  $\times 60$  air lens (numerical aperture, 0.7) (Nikon Inc., Melville, N.Y.). Cell morphology was observed using differential interference contrast microscopy, and image acquisition was made by using a Sensys chip-cooled device (CCD) camera (Photometrics). The CCD camera was driven with PMIS processing software (Photometrics), and acquired images were processed using Adobe Photoshop (version 3.0).

**Nucleotide sequence accession number.** The *A. vinelandii ampDE* operon sequence reported here has been assigned GenBank accession no. AF237388.

## RESULTS

**Isolation of strain AC28.** In *A. vinelandii* the synthesis of alginate correlates well with the transcription of the gene *algD* (5). We hypothesized that mutations in genes whose products regulate transcription of *algD* can be identified by a loss or increase in the  $\beta$ -galactosidase activity of a strain carrying an *algD*::*lacZ* transcriptional fusion such as WI12. Random Tn5

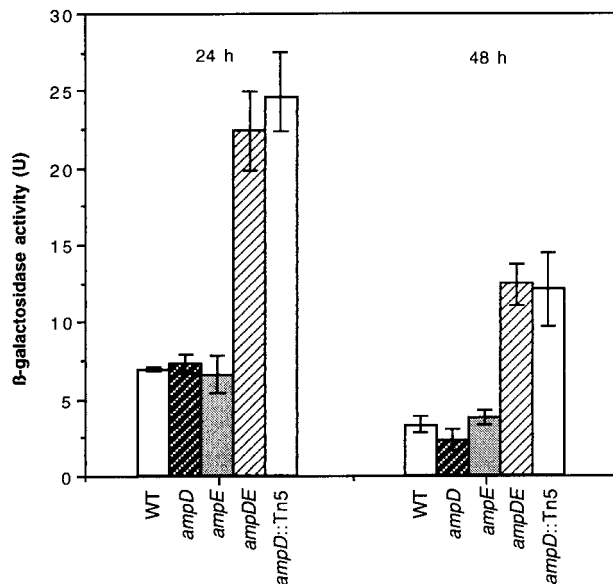


FIG. 2. *algD* transcription measured as  $\beta$ -galactosidase activity in strains WI12 (wild type [WT]), WID1 (*ampD*), WIE1 (*ampE*), WIDE1 (*ampDE*), and AC28 (*ampD*::Tn5). Cells were grown on BS solid medium and were harvested at the indicated times.

mutagenesis of strain WI12 was carried out as described in Materials and Methods. Tc<sup>r</sup> derivatives (3,200) were isolated and screened for a deep blue color on BS plates containing X-Gal. Five derivatives that were bluer than WI12 were identified (data not shown). Derivative AC28, which showed the deepest blue phenotype, was chosen for further analysis.

**Cloning and DNA sequence.** The sequence contiguous to the Tn5 insertion in AC28 was cloned to produce plasmid pK28. The DNA sequence of pK28 revealed homology to those of the *P. aeruginosa* and *E. coli ampD* genes. Southern blot analysis with pK28 as the probe led to the identification, in an *A. vinelandii* genomic library, of cosmid pSM194 carrying a 2-kb *SmaI* fragment with the *ampD* wild-type sequence. Sequence analysis of this DNA fragment revealed two open reading frames, one encoding a 187-amino-acid polypeptide (AmpD) and the second encoding a 280-amino-acid protein (AmpE). Potential Shine-Dalgarno sequences (AGGAG and GGGAG) are present upstream of both the *ampD* and *ampE* start codons. As in other bacteria, such as *E. coli* and *P. aeruginosa*, the *ampE* start codon overlaps the *ampD* TGA termination codon, suggesting that, as in *P. aeruginosa* and *E. coli*, these two genes form an operon (15, 20, 22). The predicted *A. vinelandii* AmpD and AmpE proteins exhibited 70 and 57% identity, respectively, to their homologues in *P. aeruginosa*.

**$\beta$ -Galactosidase activity of strain AC28.** To confirm and evaluate the effect of the *ampD*::Tn5 mutation on the transcription of *algD*, we compared the  $\beta$ -galactosidase activity of strain WI12 with that of strain AC28 during growth in BS. As shown in Fig. 2, transcription of *algD* in AC28, measured as  $\beta$ -galactosidase activity, was threefold higher than that in strain WI12.

**Transcription of *algD* in *ampD* and *ampE* mutants.** Since *ampD* and *ampE* genes seem to be arranged as an operon transcribed from a promoter located upstream *ampD*, it seemed likely that the *ampD*::Tn5 mutation in AC28 was polar on *ampE* expression. To investigate whether polarity on *ampE* was contributing to the increase in *algD* transcription, we con-

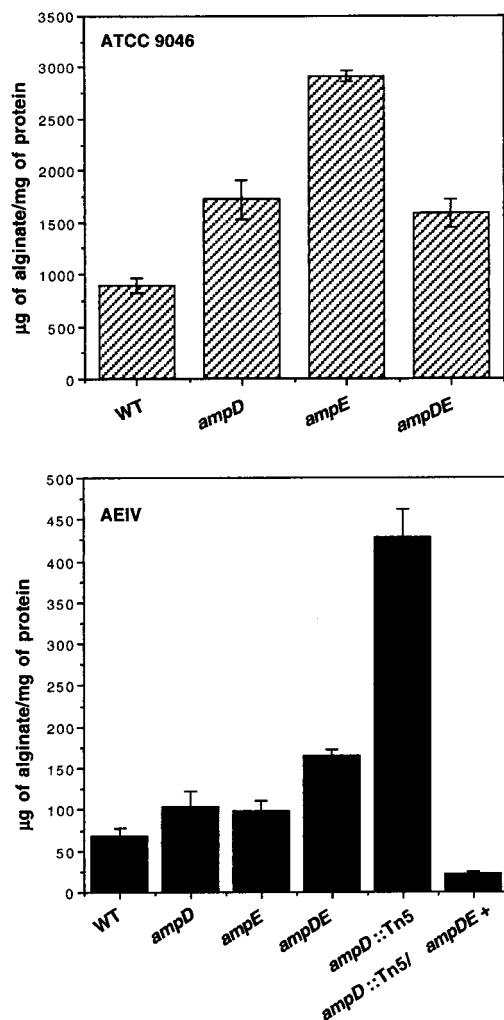


FIG. 3. Alginate production in *ampD*, *ampE*,  $\Delta$ *ampDE*, and *ampD::Tn5* derivatives of strains ATCC 9046 (top) and AEIV (bottom). Cells were grown on solid BS medium for 48 h. Alginate was separated from the cells and measured as described in Materials and Methods. WT, wild type.

structured WI12 derivatives carrying a nonpolar *ampD::Gm* mutation (WID1) and an *ampE::Sp* mutation (WIE1) and strain WIDE1 with an *ampDE* deletion. Similar to strain AC28, strain WIDE1 showed a deep blue color on X-Gal plates and had increased levels of  $\beta$ -galactosidase activity. In contrast, the *ampD* and *ampE* single mutations did not affect the transcription of *algD* (Fig. 2). These results indicate a cooperative effect of both mutations on *algD* transcription.

**Production of alginate.** In strain WI12 alginate production is reduced 75% compared to that in its parental strain, ATCC 9046; this reduction is likely due to the fact that the *lacZ* insertion is located on the 3' end of the *algD* gene, resulting in a GDP-mannose dehydrogenase with a reduced activity (5). To determine if the increased AC28  $\beta$ -galactosidase activity shown above correlated with the production of alginate, ATCC 9046 and AEIV derivatives carrying the *ampD::Gm*, *ampE::Sp*, and  $\Delta$ *ampDE::Gm* mutations were constructed. The *ampD::Tn5* insertion from AC28 was transferred by transformation to strain AEIV, thus creating an AE28 mutant. The resulting strains were found to produce more alginate than their parental strains (Fig. 3); however, the extent of such an increase

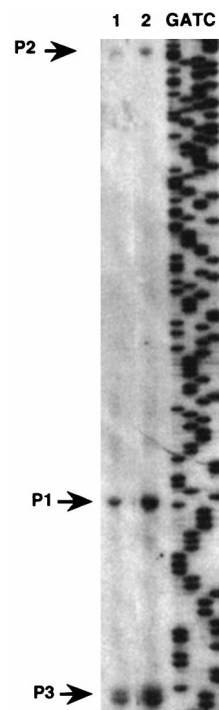


FIG. 4. Primer extension analysis of *algD* transcription in strains WI12 (lane 1) and AC28 (lane 2). RNA (50  $\mu$ g) isolated from bacterial cultures grown for 48 h in Burk's medium supplemented with 2.0% sucrose was used. Arrows, transcription initiation sites from the three *algD* promoters.

depended on the strain tested. Several attempts were made to transfer the *ampD::Tn5* mutation to the highly mucoid strain ATCC 9046 with no success. The AE28 strain exhibited a higher alginate production level than strain AEDE1, which carries a nonpolar  $\Delta$ *ampDE::Gm* mutation, whereas AE28 carries a polar *Tn5* mutation; therefore, polarity on transcription of a gene downstream of *ampE* also affecting alginate production could explain this difference. Plasmid pKT194.5, which carries a copy of the *A. vinelandii ampDE* operon, was transferred to strain AE28. As shown in Fig. 3, strain AE28/pKT194.5 produced alginate levels similar to that produced by wild-type AEIV, indicating that the high level of alginate production observed in strain AE28 was due to the absence of *ampDE* gene products.

**The *ampD::Tn5* mutation increases transcription of *algD* from its three promoters.** As in strain ATCC 9046, transcription of *algD* in strain WI12 is initiated from three sites (Fig. 4). We tested whether the increase in *algD* transcription in strain AC28 was caused by specifically increasing initiation from one of these promoters. Primer extension analysis using RNA isolated from strains WI12 and AC28 was carried out. As shown in Fig. 4, the *ampD::Tn5* mutation increased transcription of *algD* from its three promoters.

**Effect of *ampD* and *ampE* mutations on the morphology of *A. vinelandii*.** The bacterial cell wall determines the shape of the cell (34). As *ampD* mutants are impaired in peptidoglycan recycling, the effect of the *ampD* and *ampE* mutations on cell shape was investigated. Microscopic examination of strains WID1, WIE1, WIDE1, and AC28 grown on BS plates was carried out. As shown in Fig. 5, the parental WI12 strain cells had a normal rod shape and were 3 to 5  $\mu$ m long. The nonpolar *ampD* mutation produced cells similar to those found in WI12, whereas the *ampE* mutation produced spherical cells. Cultures



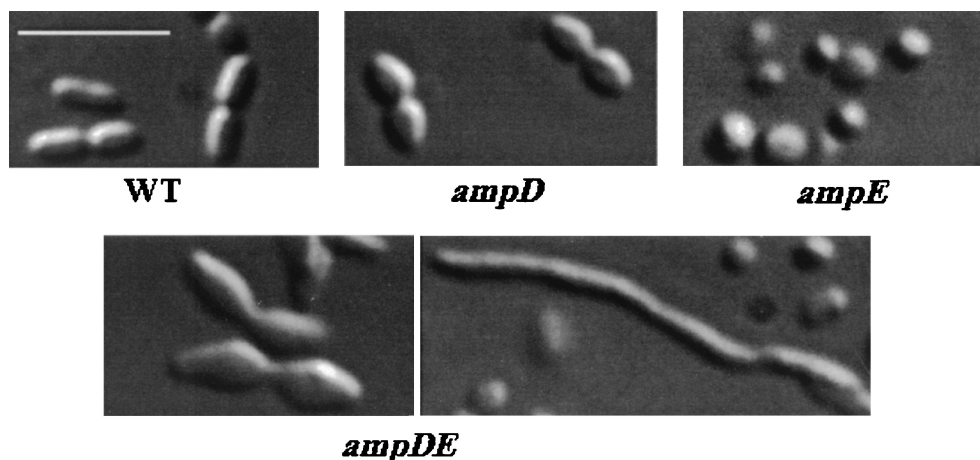


FIG. 5. Effect of mutations in the *ampDE* operon on cell morphology in *A. vinelandii*. All photographs were captured with a  $\times 60$  air lens. Bar, 10  $\mu\text{m}$ . WT, wild type.

derived from AC28 and WIDE1 mutants showed a heterogeneous cell morphology; they produced filamented and spherical cells. In addition they produced cells whose poles have an arrow shape, and the septa between dividing cells are abnormal, suggesting a failure to produce a normal septum (Fig. 5). The effect of the *ampDE* mutations on cell morphology was less dramatic when the mutants were grown on liquid medium (data not shown).

**Effect of the *ampDE* mutation on encystment.** Transport of wall-derived peptides into the cell has been proposed to have a signaling role in the initiation of sporulation in *B. subtilis* (35). We tested the effect of the *ampDE* mutation on encystment. The formation of desiccation-resistant cells in WI12 and AC28 cultures was measured. Strain WI12 has a frequency of cyst formation of about 5.0%, while mutant AC28 has a frequency of less than  $10^{-6}$ . After induction, vegetative cells divide to give small rounded cells that eventually become mature cysts. As expected, WI12 cells induced for encystment on *n*-butanol plates produced small rounded cells; in contrast, AC28 cells induced for encystment had the abnormal morphology found in vegetative cultures of this mutant (data not shown).

## DISCUSSION

The aim of this study was to identify genes whose products participate in the control of alginate production. The isolation of mutants in which *algD* transcription was increased led us to the identification of the *A. vinelandii ampD* and *ampE* genes. In *P. aeruginosa* and other gram-negative bacteria, the *ampDE* operon participates in a pathway for recycling the cell wall. This recycling pathway was proposed to regulate other functions besides  $\beta$ -lactamase induction (33, 34).

This study presents evidence suggesting that, in *A. vinelandii*, peptidoglycan recycling participates in the regulation of alginate production. Similar to the effect of the *ampDE* mutation on transcription of *ampC*, inactivation of the *ampDE* operon in *A. vinelandii* resulted in an increase in *algD* transcription accompanied by an increase in alginate production. Neither *ampD* nor *ampE* single mutations seemed to affect *algD* transcription although they resulted in an increased alginate production. Similarly, in an *algR* mutant, alginate production was reduced 50% despite the fact that *algD* transcription was not affected (30). This suggests the presence of another regulated point, besides the *algD* promoter, which also determines the

extent of alginate production. The *ampD* and *ampE* mutations could affect this regulation point, resulting in higher alginate levels.

In *A. vinelandii* the increase in *algD* expression observed in the *ampDE* mutants might be caused by accumulation of the AmpD substrate. We propose that inactivation of *ampE* also contributes to the accumulation of the anhydromuropeptide in the cytoplasm. This hypothesis implies that AmpE also participates in the peptidoglycan recycling pathway. The fact that *ampE* and *ampD* genes constitute an operon supports this hypothesis. AmpE might be involved in regulating the extent of cell wall breakdown, slowing down the lytic process by controlling the activity of lytic transglycosylases. In *E. coli* three lytic transglycosylases and two endopeptidases are present in the periplasm or bound to the cytoplasmic membrane with the active sites oriented toward the periplasm (40). If AmpE was involved in cell wall breakdown, lysis of the cell wall would increase in the absence of AmpE, with the subsequent increase of anhydromuropeptides in the cytoplasm, which in the absence of AmpD accumulate and serve as a regulatory signal.

The *ampDE* mutation increased the transcription of *algD* from its three initiation sites. We have recently reported that the two-component global regulatory system GacSA also controls *algD* transcription from its three sites (6); thus, it is possible that the GacSA system participates in the signal transduction pathway turned on in the *ampDE* mutants. Accumulation of murein precursors in the *A. vinelandii ampDE* mutants might increase *algD* transcription directly or indirectly by activating an AmpR-like regulator.

This study shows that mutations in the *ampDE* operon also affected cell shape. We found that the *ampE* mutation produced rounded cells. In *E. coli*, strains with mutations in *rodA*-*pbpA* or the *mre* region (41, 43) are defective in the elongation process and grow as spherical cells, suggesting that in the *ampE* mutant cell elongation may be affected. The *ampDE* mutations produced, in addition to rounded cells, filamented cells of different lengths, a phenotype similar to those of *E. coli pbpB* mutants. In *E. coli*, PBP3, the product of gene *pbpB*, is involved in septum formation (4). Temperature-sensitive *pbpB* mutants stop dividing at high temperature and form long filaments (44). Nothing is known about the regulatory mechanisms controlling cell wall elongation and septum formation; however, it was suggested that changes in the cytoplasmic concentration of anhydromuropeptides could play a role (33). In

this context, the morphological aberrations observed in the double mutant *ampDE* could be due to the presence of high levels of anhydromuropeptide which, in turn, deregulates the events of cell wall elongation and septum formation. Since the cell wall determines the shape of a cell (34), this result indicates that cell wall recycling has important implications in determining cell shape.

Upon induction of encystment, *A. vinelandii* vegetative cells divide to give two small rounded cells, which are later surrounded by an inner coat, the intine, and a thick laminated exine (38). Alginate is a component of the intine and exine layers. *A. vinelandii algU* or *algK* mutants are unable to produce alginate and therefore to form a mature cyst; upon encystment, these mutants form rounded cells lacking the intine and exine layers (26, 29). A mutation in *algR* did not impair alginate production but caused a cyst-defective phenotype, producing cysts lacking the intine layer (30). The *ampDE* mutant reported here is impaired in encystment. Upon conditions for induction of encystment, cells of this mutant remained as vegetative cells. Thus, encystment seems to be blocked at the first step. This result suggests that the level of muropeptides in the cytoplasm could be a signal necessary for *A. vinelandii* cells to undergo the differentiation process.

Finally, alginate synthesis also occurs independently of encystment; this polysaccharide is normally produced by vegetative cells not undergoing this differentiation process. This indicates that these two cellular processes can be separated and is in agreement with the finding that the *ampDE* mutation enhanced alginate synthesis but abolished encystment.

#### ACKNOWLEDGMENTS

This work was supported by grant 27767-N from CONACyT to G.E. The microscopic study was carried out with an equipment acquired with grants 27698-N and 27640-N from CONACyT and grant IN212298 from DGAPA to F. Sánchez and C. Quinto. L.C. was supported by installation grant for young scientists I29972-N from CONACyT.

We thank J. Guzmán for technical support and O. Geiger and M. Villanueva for reviewing the manuscript.

#### REFERENCES

- Alexeyev, M. F., I. Shokolenko, and T. P. Croughan. 1995. Improved antibiotic-resistance gene cassettes and omega elements for *Escherichia coli* vector construction and in vitro deletion/insertion mutagenesis. *Gene* **160**: 63–67.
- Bagdasarian, M., L. Lurz, B. Ruckert, F. C. Franklin, M. M. Bagdasarian, J. Frey, and K. N. Timmis. 1981. Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF 1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene* **16**:237–247.
- 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.
- Botta, G. A., and J. T. Park. 1981. Evidence for involvement of penicillin-binding protein 3 in murein synthesis during septation but not during cell elongation. *J. Bacteriol.* **145**:333–340.
- Campos, M. E., J. M. Martínez-Salazar, L. Lloret, C. Nuñez, G. Espín, and G. Soberón-Chávez. 1996. Cloning and characterization of the gene coding for GDP-mannose dehydrogenase (*algD*) from *Azotobacter vinelandii*. *J. Bacteriol.* **178**:1793–1799.
- Castañeda, M., J. Guzmán, S. Moreno, and G. Espín. 2000. GacS sensor kinase regulates alginate and poly- $\beta$ -hydroxybutyrate production in *Azotobacter vinelandii*. *J. Bacteriol.* **182**:2624–2628.
- Darzens, A., and A. M. Chakrabarty. 1984. Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa*. *J. Bacteriol.* **159**:9–19.
- de Lorenzo, V., M. Herrero, V. Jakubzik, and K. N. Timmis. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol.* **172**:6568–6572.
- Ertesvag, H., H. K. Hoidal, I. K. Hals, A. Rian, B. Doseth, and S. Valla. 1995. A family of modular type mannuronan C-5-epimerase genes controls alginate structure in *Azotobacter vinelandii*. *Mol. Microbiol.* **16**:719–731.
- Fellay, R., J. Frey, and H. Krisch. 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of gram-negative bacteria. *Gene* **52**:147–154.
- Figurski, D., and R. D. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. USA* **76**:1648–1652.
- Goodell, E. W. 1985. Recycling of murein by *Escherichia coli*. *J. Bacteriol.* **163**:305–310.
- Goodell, E. W., and U. Schwarz. 1985. Release of cell wall peptides into culture medium by exponentially growing *Escherichia coli*. *J. Bacteriol.* **162**: 391–397.
- Hanahan, D. 1983. Studies on transformation of *E. coli*. *J. Mol. Biol.* **166**: 557–580.
- Honoré, N., M. H. Nicolas, and S. T. Cole. 1989. Regulation of enterobacterial cephalosporinase production: the role of a membrane bound sensory transducer. *Mol. Microbiol.* **3**:1121–1130.
- Jacobs, C., L. Huang, E. Bartowsky, S. Normark, and J. T. Park. 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for  $\beta$ -lactamase induction. *EMBO J.* **13**:4684–4694.
- Jacobs, C., B. Joris, M. Jamin, K. Klarsov, J. V. Beeumen, D. Mengin-Lecreulx, J. van Heijenoort, J. T. Park, S. Normark, and J. M. Frère. 1995. AmpD, essential for both  $\beta$ -lactamase regulation and cell wall recycling, is a novel cytosolic *N*-acetylmuramyl-L-alanine amidase. *Mol. Microbiol.* **15**:553–559.
- Jacobs, C., J. M. Frère, and S. Normark. 1997. Cytosolic intermediates for cell wall biosynthesis and degradation control inducible  $\beta$ -lactam resistance in gram-negative bacteria. *Cell* **88**:823–832.
- Kennedy, C., R. Gamal, R. Humprey, J. Ramos, K. Brigle, and D. Dean. 1986. The *nifH*, *nifM*, and *nifN* genes of *Azotobacter vinelandii*: characterization by Tn5 mutagenesis and isolation from pLARF1 gene bank. *Mol. Gen. Genet.* **205**:318–325.
- Langae, T. Y., M. Dargis, and A. Huletasy. 1998. An *ampD* gene in *Pseudomonas aeruginosa* encodes a negative regulator of AmpC  $\beta$ -lactamase expression. *Antimicrob. Agents Chemother.* **42**:3296–3300.
- Lindberg, F., S. Lindquist, and S. Normark. 1987. Inactivation of the *ampD* gene causes semiconstitutive overproduction of the inducible *Citrobacter freundii*  $\beta$ -lactamase. *J. Bacteriol.* **169**:1923–1928.
- Lindquist, S., M. Galleni, F. Lindberg, and S. Normark. 1989. Signalling proteins in enterobacterial AmpC  $\beta$ -lactamase regulation. *Mol. Microbiol.* **3**:1091–1102.
- Lloret, L., R. Barreto, R. León, S. Moreno, J. Martínez-Salazar, G. Espín, and G. Soberón-Chávez. 1996. Genetic analysis of the transcriptional arrangement of *Azotobacter vinelandii* alginate biosynthetic genes: identification of two independent promoters. *Mol. Microbiol.* **21**:449–457.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
- Martínez-Salazar, J. M., S. Moreno, R. Nájera, C. Boucher, G. Espín, G. Soberón-Chávez, and V. Deretic. 1996. Characterization of the genes coding for the putative sigma factor AlgU and its negative regulators MucA, MucB, MucC, and MucD in *Azotobacter vinelandii* and evaluation of their role in alginate biosynthesis. *J. Bacteriol.* **178**:1800–1808.
- Mejía-Ruiz, H., S. Moreno, J. Guzmán, R. Nájera, R. León, G. Soberón-Chávez, and G. Espín. 1997. Isolation and characterization of an *Azotobacter vinelandii algK* mutant. *FEMS Microbiol. Lett.* **156**:101–106.
- Mejía-Ruiz, H., J. Guzmán, S. Moreno, G. Soberón-Chávez, and G. Espín. 1997. The *Azotobacter vinelandii alg8* and *alg44* genes are essential for alginate synthesis and can be transcribed from an *algD*-independent promoter. *Gene* **199**:271–277.
- Miller, J. H. 1972. Experiments in molecular genetics, p. 431–435. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Moreno, S., J. Guzmán, R. Nájera, G. Soberón-Chávez, and G. Espín. 1998. Role of the alternative  $\sigma^E$  factor AlgU in encystment of *Azotobacter vinelandii*. *J. Bacteriol.* **180**:2766–2769.
- Núñez, C., S. Moreno, G. Soberón-Chávez, and G. Espín. 1999. The *Azotobacter vinelandii* response regulator AlgR is essential for cyst formation. *J. Bacteriol.* **181**:141–148.
- Page, W. J., and H. L. Sadoff. 1975. Relationship between calcium and uronic acids in the encystment of *Azotobacter vinelandii*. *J. Bacteriol.* **122**:145–151.
- Park, J. T. 1993. Turnover and recycling of the murein sacculus in oligopeptide permease-negative strains of *Escherichia coli*: indirect evidence for an alternative permease system and monolayered sacculus. *J. Bacteriol.* **175**:7–11.
- Park, J. T. 1995. Why does *Escherichia coli* recycle its wall peptides? *Mol. Microbiol.* **17**:421–426.
- Park, J. T. 1996. The murein sacculus, p. 48–57. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umberger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
- Perego, M., C. F. Higgins, S. R. Pearce, M. P. Gallagher, and J. A. Hoch. 1991. The oligopeptide transport system of *Bacillus subtilis* plays a role in the initiation of sporulation. *Mol. Microbiol.* **5**:173–185.

36. **Pindar, D. F., and C. Bucke.** 1975. The biosynthesis of alginic acid by *Azotobacter vinelandii*. *Biochem. J.* **152**:617–622.
37. **Rehm, H. A., H. Ertesvag, and S. Valla.** 1996. A new *Azotobacter vinelandii* mannuronan C-5 epimerase gene (*algG*) is part of an *alg* gene cluster physically organized in a manner similar to that in *Pseudomonas aeruginosa*. *J. Bacteriol.* **178**:5884–5889.
38. **Sadoff, H. L.** 1975. Encystment and germination in *Azotobacter vinelandii*. *Bacteriol. Rev.* **39**:516–539.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Shockman, G. D., and J. V. Høltje.** 1994. Bacterial cell wall. *New Compr. Biochem.* **27**:131–166.
41. **Spratt, B. G., A. Boyd, and N. Stoker.** 1980. Defective and plaque forming lambda transducing bacteriophage carrying penicillin-binding protein-cell shape genes: genetic and physical mapping and identification of gene products from the *lip-dacA-rodA-pbpA-leuS* region of *Escherichia coli* chromosome. *J. Bacteriol.* **143**:569–581.
42. **Vazquez, A., S. Moreno, J. Guzmán, A. Alvarado, and G. Espín.** 1999. Transcriptional organization of the *Azotobacter vinelandii* *algGXLVIFA* genes: characterization of *algF* mutants. *Gene* **232**:217–222.
43. **Wachi, M., M. Doi, S. Tamaki, W. Park, S. Nakajima-Iijima, and M. Matsubashi.** 1987. Mutant isolation and molecular cloning of *mre* genes, which determine cell shape, sensitivity to mecillinam, and amount of penicillin-binding proteins in *Escherichia coli*. *J. Bacteriol.* **169**:4935–4940.
44. **Walker, J., A. Kovarik, J. Allan, and R. Gustafson.** 1975. Regulation of bacterial cell division: temperature-sensitive mutants of *Escherichia coli* that are defective in septum formation. *J. Bacteriol.* **123**:693–703.
45. **Xie, Z.-D., C. D. Hershberger, S. Shankar, R. W. Ye, and A. M. Chakrabarty.** 1996. Sigma factor–anti-sigma factor interaction in alginate synthesis: inhibition of AlgT by MucA. *J. Bacteriol.* **178**:4990–4996.