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The Global Regulators GacA and σ^S Form Part of a Cascade That Controls Alginate Production in *Azotobacter vinelandii*

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Transcription of the *Azotobacter vinelandii algD* gene, which encodes GDP-mannose dehydrogenase (the rate-limiting enzyme of alginate synthesis), starts from three sites: p1, p2, and p3. The sensor kinase GacS, a member of the two-component regulatory system, is required for transcription of *algD* from its three sites during the stationary phase. Here we show that *algD* is expressed constitutively throughout the growth cycle from the p2 and p3 sites and that transcription from p1 started at the transition between the exponential growth phase and stationary phase. We constructed *A. vinelandii* strains that carried mutations in *gacA* encoding the cognate response regulator of GacS and in *rpoS* coding for the stationary-phase σ^S factor. The *gacA* mutation impaired alginate production and transcription of *algD* from its three promoters. Transcription of *rpoS* was also abolished by the *gacA* mutation. The *rpoS* mutation impaired transcription of *algD* from the p1 promoter and increased it from the p2 σ^E promoter. The results of this study provide evidence for the predominant role of GacA in a regulatory cascade controlling alginate production and gene expression during the stationary phase in *A. vinelandii*.

Azotobacter vinelandii is a nitrogen-fixing soil bacterium that undergoes differentiation to form desiccation-resistant cysts and produces two polymers of industrial importance: alginate and poly-β-hydroxybutyrate (PHB).

A. vinelandii has been shown to posses an alginate biosynthetic gene cluster organized in three operons (5, 25, 29, 30, 49), one of which transcribes *algD*, which encodes GDP-mannose dehydrogenase, the key enzyme of the alginate biosynthetic pathway. The *algUmucABCD* cluster has been characterized in *A. vinelandii* and in *Pseudomonas aeruginosa* and has been shown to control alginate production (28, 34, 37, 44, 45, 53). It has been shown for *P. aeruginosa* that the activity of the alternative sigma factor σE (AlgU) encoded by *algU* is negatively regulated by the anti-sigma factor MucA (9, 10, 16, 27, 45, 54) and in an indirect manner by MucB (27). In several bacterial species, σ^E regulates expression of functions related to the extracytoplasmic compartments (32). In *A. vinelandii*, transcription of *algD* can initiate at three promoters, one of which (p2) is regulated by σ^E (28, 34) but presumably in an indirect manner (37).

The global two-component GacS/GacA system is conserved in a variety of gram-negative bacteria. In *Erwinia carotovora* and some *Pseudomonas* species, it controls the expression of genes involved in secondary metabolism, phytopathogenesis, and quorum sensing (7, 8, 11, 15, 20, 24, 40, 41). In *Pseudomonas syringae* B728a, *gacA* and *gacS* mutations negatively affect alginate production and *algD* expression (52).

The GacS histidine kinase controls alginate production in *A. vinelandii*. In *gacS* mutants transcription of *algD* is significantly reduced during exponential growth and abolished in the stationary phase (6). Regulation of alginate synthesis by GacS during the stationary phase was shown to be exerted on *algD* transcription from its three promoters (6).

In *Escherichia coli* and other bacteria, the alternative sigma factor σ^S (RpoS) functions as a global regulator and is responsible for the activation of many genes expressed mainly during the stationary phase and under various stress conditions (18). One way in which GacA regulates gene expression in *Pseudomonas fluorescens* is by influencing accumulation of the σ^S factor (50). In *P. aeruginosa*, σ^S controls the production of virulence factors, such as exotoxin A, pyocyanin, and alginate in an alginate-overproducing strain (48). A relationship between σ^S and quorum sensing has also been reported in *P. aeruginosa* (22, 51).

In *E. coli* transcription of *rpoS* in exponentially growing cells is dependent on BarA (35). BarA was recently identified as the cognate sensor kinase of UvrY, the *E. coli* GacA homologue (39). As GacS/GacA influences the level of σ^S in several bacterial species, expression of *algD* in *A. vinelandii* was proposed to be regulated by σ^S (6). In agreement with this proposition, the *A. vinelandii algD*-p1 promoter has the −10 sequence CTA TAAT and also has an intrinsic DNA curvature observed in promoters preferentially recognized by σ^S (12, 13, 38).

Most of the two-component systems are composed of a transmembrane histidine phosphokinase that senses environmental signals and a cytoplasmic response regulator that activates transcription upon phosphorylation by the sensor (19, 47).

This study reports the identification and characterization of the *A. vinelandii gacA* gene, which encodes the GacS cognate response regulator, and *rpoS*, which encodes the σ^S factor. Our data show that entering into the stationary phase results in expression of *rpoS* and of *algD* from its p1 promoter and that

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
A. vinelandii strains		
ATCC 9046	Highly mucoid, wild type	ATCC
JM3	ATCC 9045 with a *gacA*::Gm mutation	This work
CNS59	ATCC 9046 with a *rpoS*::Sp mutation	This work
E. coli strain		
DH5α	*supE44* Δ*lacU169 hsdR17 recA1 endA1 gyrA96 hi-1 relA1*	GIBCO-BRL
Plasmids		
pBluescript KS(+)		Stratagene
pKT230	Broad-host-range vector, Km^r Sm^r	2
pHP45Ω-Sp		14
pBSL141-Gm		1
pSMU1886	pCP13 cosmid vector containing 25 kb of *A. vinelandii* DNA, including *gacA*	This work
pSAFA1	pBluescript KS(+) carrying the *gacA* gene cloned by PCR	This work
pSAFA2	pBluescript KS(+) carrying a 3.0-kb *Cla*I fragment with *gacA*	This work
pSAFA3	pSAFA2 derivative with a *gacA*::Sp mutation	This work
pSAFA4	pKT230 vector carrying the *gacA* gene cloned by PCR	This work
pCNS59	pBluescript KS(+) carrying a 0.8-kb fragment with *rpoS*	This work
pSMS7	pCNS59 derivative with an *rpoS*::Sp mutation	This work

a mutation in *gacA* abrogates transcription of *algD* and *rpoS*, indicating the predominant role of GacA in a regulatory cascade that controls gene expression in the stationary phase and alginate production in *A. vinelandii*.

MATERIALS AND METHODS

Microbiological procedures. Bacterial strains and plasmids used are listed in Table 1. Medium and growth conditions were as follows: *A. vinelandii* was grown at 30°C in Burk's nitrogen-free salts medium supplemented with 2% sucrose (21). *E. coli* strain DH5α was grown on Luria-Bertani medium (31) at 37°C. Antibiotic concentrations used (in micrograms per milliliter) for *A. vinelandii* and *E. coli,* respectively, were as follows: tetracycline, 20 and 20; kanamycin, 5 and 30; rifampin, not used and 20; ampicillin, not used and 100; nalidixic acid, 20 and 20; spectinomycin, 100 and 100; streptomycin, 2 and 20; and gentamicin, 1.5 and 10. *A. vinelandii* transformation was carried out as previously described (3).

Alginate and PHB production was determined as previously described (30, 46). All measurements were done in triplicate. Protein concentration was determined by the Lowry method (26).

Nucleic acid procedures. RNA and DNA isolation and cloning, Southern blotting, and random primer procedures were carried out as described earlier (42). Plasmids pSAFA2 and pCNS59 were used to determine the nucleotide sequences reported in this study. DNA sequencing was done with the Thermosequenase sequencing kit by the dideoxy-chain termination method of Sanger et al. (43). Primer extension of *algD* and *algU* was carried out as previously described (5, 37). Reactions were performed with a primer extension system (Amersham) as instructed by the manufacturer.

Northern blot analysis. Total RNA was extracted from the ATCC 9046 and JM3 strains using a High-Pure RNA Isolation Kit (Roche) and was quantified spectrophotometrically by measuring optical density at 260 nm. For Northern analysis 10 μg of RNA was loaded per lane. As loading and transfer controls, all blots were reprobed with a probe specific to 16S rRNA derived from plasmid pKK3535 (4).

Cloning of *A. vinelandii gacA* and *rpoS* genes. Oligonucleotides gacA1 (5′-GATTAAGGTGCTGGTGGTCGACC-3′) and gacA2 (5′-GCGGTGCCGTACCAGCTACGGCGG-3′) and total DNA from *P. aeruginosa* PAO1 were used to isolate by PCR a fragment containing the *P. aeruginosa gacA* gene (40). This fragment was used as probe to identify a cosmid clone denoted pSMU1886, which was derived from an *A. vinelandii* genomic library, and contained a 3-kb *Cla*I fragment that hybridized to the *gacA* probe. This 3-kb *Cla*I fragment was cloned into the pBluescript KS(+) vector (Stratagene) to yield plasmid pSAFA2 (Fig. 1). Oligonucleotides jsf2 (5′-TTGCCCACCTCCCGGGTGG-3′) and jsf3 (5′-GCAGGGATCCAGAAAAGCCG-3′) were used to isolate by PCR a fragment containing the *gacA* gene. This fragment was cloned into plasmid pKT230 (2) to produce pSAFA4 (Fig. 1).

Oligonucleotides rpoS5 (5′-TTGGACGCAACGCAGCTGTATC-3′) and rpoS3 (5′-CTGGATCTGACGAACCCGCTC-3′) were designed based on the *P. aeruginosa rpoS* sequence and correspond to a σ^S conserved region among various species. Total DNA from *A. vinelandii* ATCC 9046 and these oligonucleotides were used to clone by PCR a 756-bp fragment that was ligated into the pBluescript KS(+) vector to yield plasmid pCNS59. Sequence analysis of this fragment confirmed the presence of the *A. vinelandii rpoS* gene.

Construction of *gacA* and *rpoS* mutants. Plasmid pSAFA2 (Fig. 1), which carries a 3.0-kb *Cla*I DNA fragment including *gacA*, was used to construct a *gacA*::Gm mutation. A 0.8-kb fragment containing a gentamicin cassette from plasmid pBSL141-Gm (1) was inserted into the unique *Stu*I site to create a *gacA*::Gm mutation within the codon for amino acid residue 137 of GacA. The resultant plasmid pSAFA3 (Fig. 1), which is unable to replicate in *A. vinelandii*, was introduced into strain ATCC 9046. Strain JM3, a Gm^r Ap^s transformant, was selected. Plasmid PCNS59 was used to construct an *rpoS* mutation. A 2-kb fragment containing a Ω-spectinomycin cassette from plasmid pHP45Ω-Sp (14) was inserted into the unique *Stu*I site to create the *rpoS*::Sp mutation within the codon for amino acid residue 130 of RpoS. The resultant plasmid pSMS7, which is unable to replicate in *A. vinelandii*, was introduced into strain ATCC 9046. Strain CNS59, a Sp^r Ap^s transformant, was selected and confirmed by Southern blot analysis to carry the *rpoS*::Sp mutation (data not shown).

Nucleotide sequence accession number. The nucleotide sequences of the *gacA* and *rpoS* genes reported here have been assigned GenBank accession numbers AF382827 and AY029155, respectively.

RESULTS AND DISCUSSION

DNA sequence of *A. vinelandii gacA* gene. The *A. vinelandii* GacS sensor kinase was previously shown to play a role as a positive regulator of polymer synthesis, since a *gacS* mutation significantly reduced alginate and PHB production. To further study regulation of alginate production by the global two-component GacSA system, we cloned, as described in Materials and Methods, an *A. vinelandii* sequence that hybridized to *P. aeruginosa gacA*. DNA sequence analysis of this fragment revealed an open reading frame encoding a 214-amino-acid polypeptide (GacA). The identity of *A. vinelandii* GacA was 85% with GacA present in the following *Pseudomonas* species: *P. syringae* (41), *Pseudomonas viridiflava* (24), *P. fluorescens* (8), *Pseudomonas aureofaciens* (7), and *Pseudomonas tolaasii* (17). Following *gacA,* a partial *orf* gene encoding 22 amino acids sharing similarity to UvrC, an exonuclease that participates in DNA repair after UV damage (33), was found. A potential Shine-Dalgarno sequence (AGGAG) is present upstream of

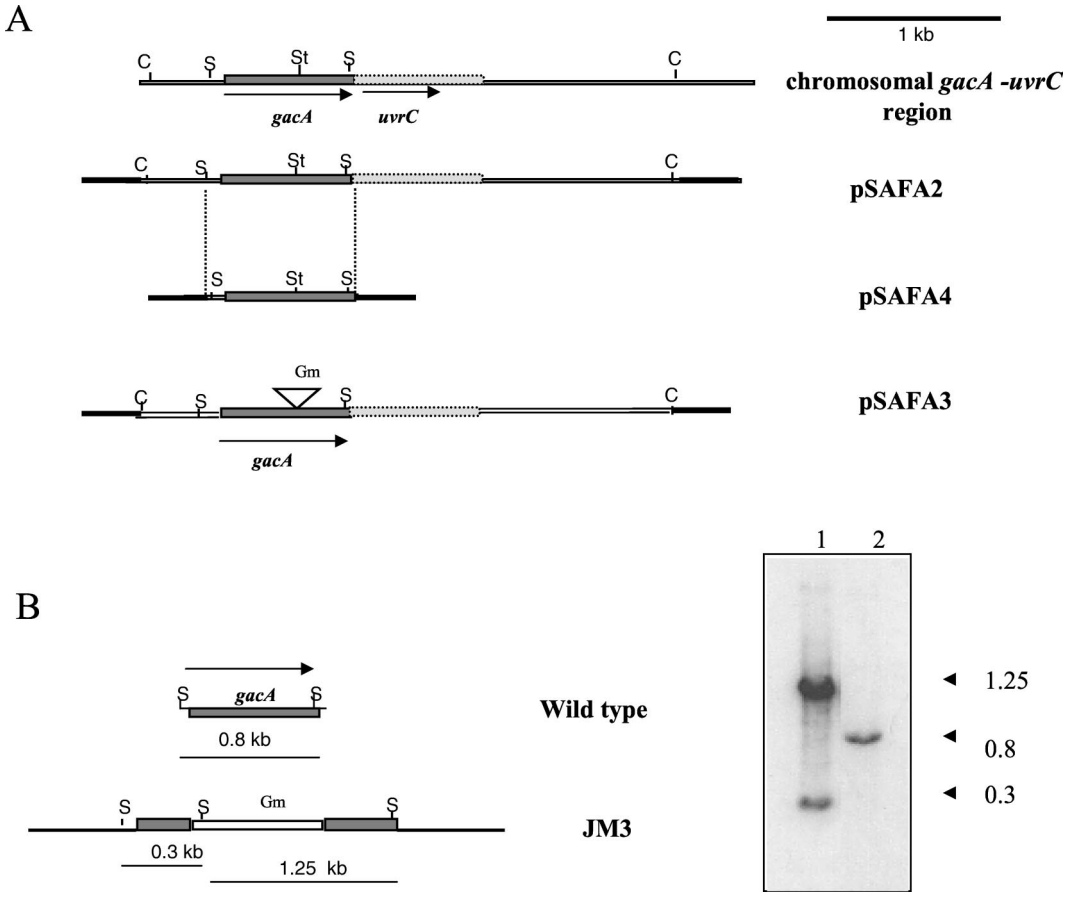

FIG. 1. (A) Physical map of the *A. vinelandii* chromosomal *gacA-uvr* region and plasmids constructed in this study. Arrows indicate direction of transcription. Antibiotic resistance cassette is represented by the inverted triangle. Vector sequences are represented by black bars. (B) Physical map of insertional inactivation of the *gacA* gene in *A. vinelandii* ATCC 9046. Southern blot hybridization of total genomic DNA digested with *Sal*I endonuclease, with the 0.8-kb *Sal*I fragment as probe. Lane 1, ATCC 9046; lane 2, JM3. Abbreviations: S, *Sal*I; C, *Cla*I; St, *Stu*I.

the *gacA* start codon. As in other bacteria, the *uvrC* start codon overlaps the *gacA* TGA termination codon (11, 33, 40), suggesting that these two genes form an operon. As with other response regulators, GacA contains two highly conserved aspartate residues, Asp8 and the predicted phospho-accepting aspartate Asp54.

Alginate and PHB production is under GacA control. As the GacS cognate response regulator, GacA was expected to act as positive regulator of biosynthesis of both alginate and PHB. Strain JM3, an ATCC 9046 derivative carrying a *gacA*::Gm mutation, was constructed as described in Materials and Methods and was shown by Southern blot analysis to carry the *gacA*::Gm mutation (Fig. 1B). Strain JM3 was unable to produce alginate and PHB (Table 2), confirming that GacA is an activator of the synthesis of these polymers.

UvrC is involved in resistance to UV in both *Pseudomonas* species and *E. coli*. Strain JM3 was more sensitive to UV light than was wild-type strain ATCC 9046 or the *gacS* mutant (data not shown). Plasmid pSAFA4 restored to the JM3 mutant the ability to produce alginate and PHB (Table 2) but did not restore resistance to UV, suggesting that the *gacA* mutation exerted polarity on *uvrC* transcription and that *gacA* and *uvrC* are organized as an operon. This data also confirmed that the inability to produce alginate and PHB is caused by the absence of the *gacA* gene product. These results provide genetic evidence supporting the conclusion that GacA is the cognate response regulator of GacS.

Growth-phase-dependent expression of *algD* and its control by GacA. In previous studies, transcription of *algD* from its three promoters was documented by primer extension experiments carried out in stationary-phase cells collected after 48 h of growth in Burk's sucrose medium (6, 34, 36). We also reported that a *gacS* mutation abolished transcription of *algD*

TABLE 2. Alginate and PHB production in *A. vinelandii* strains[a]

Strain	Alginate concn (mg/mg of protein)	PHB concn (μg/mg of protein)
ATCC 9046	1.93 ± 0.3	460 ± 24
JM3	<1.0	<10.0
JM3/pSAFA3	1.96 ± 0.3	455 ± 21
CNS59	1.70 ± 0.3	ND[b]

[a] Alginate and PHB concentrations were determined in cells grown for 48 h in Burk's liquid medium supplemented with 2% sucrose. Values are means ± standard errors of the means.

[b] ND, not determined.

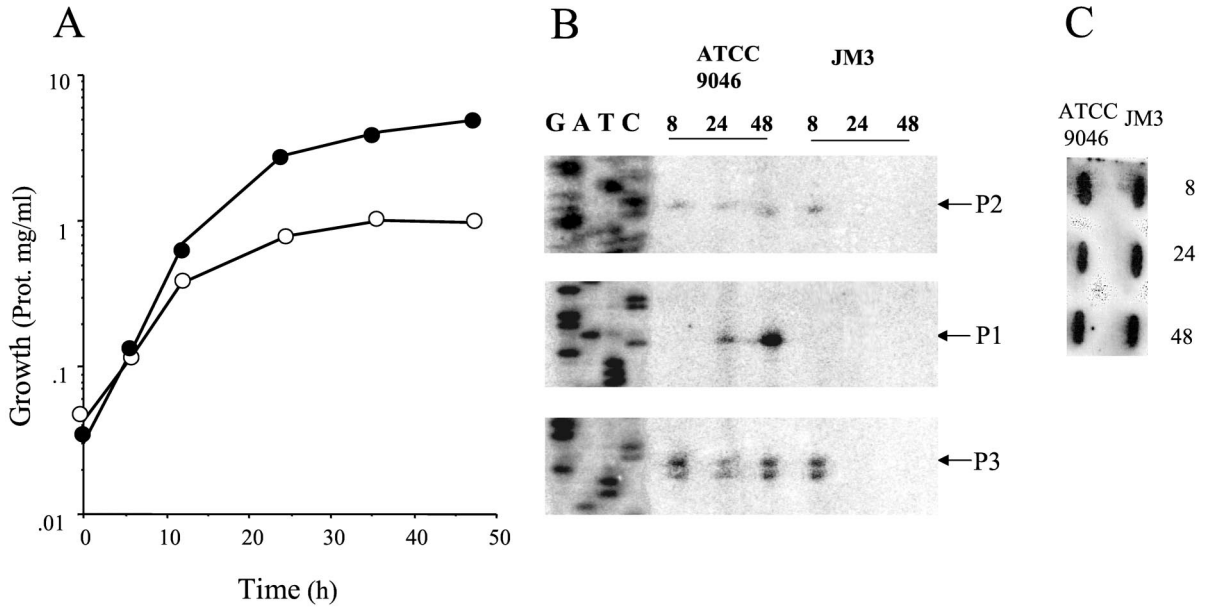

FIG. 2. Growth and primer extension analysis of *algD*. (A) Growth of ATCC 9046 (solid circles) and JM3 strains (empty circles) in Burk's sucrose medium. (B) Primer extension at 8 (lane 1), 24 (lane 2), and 48 h (lane 3) incubation in Burk's sucrose medium. (C) Hybridization of a sample of the RNA (10 μg) used as template for the primer extension with a probe specific for 16S rRNA over 8, 24, and 48 h (4).

during the stationary phase; however, during exponential growth some transcription of *algD* (determined by β-galactosidase activity with an *algD-lacZ* fusion) was detected in a *gacS* mutant (6). To further study the control of *algD* expression in *A. vinelandii*, the transcriptional induction kinetic of *algD* was determined by primer extension in cells of ATCC 9046 and the *gacA* mutant JM3 throughout a growth cycle on liquid Burk's sucrose medium (Fig. 2A). A reduction of growth was observed in strain JM3, suggesting a GacA requirement for the control of factors contributing to optimal growth. In the exponential phase, *algD* transcription initiated from the p2 and p3 but not from the p1 promoter. Transcription from the p1 promoter started at the transition between exponential growth and stationary phase and increased when cells reached the stationary phase (Fig. 2). This result is in agreement with the hypothesis that p1 is a σ^S-dependent promoter.

The effect of the *gacA* mutation on transcription of the *algD* throughout a growth cycle is shown in Fig. 2B. Similar to results for the wild type, primer extension products corresponding to the p2 and p3 promoter but not from p1 were detected in strain JM3 in exponentially growing cells (Fig. 2). This is an unexpected result, since in the *gacS* mutant, transcription of *algD* (measured as β-galactosidase activity with an *algD-lacZ* fusion) was reduced during exponential growth (6). However, similar to the result reported with the *gacS* mutant (6), during the stationary phase no primer extension products corresponding to the three promoters were detected in this *gacA* mutant. This result indicates that the GacS/GacA system is essential for activation of the three *algD* promoters during the stationary phase. These data also imply that control of alginate synthesis is to some extent growth phase dependent.

***algD*-p1 is a σ^S-dependent promoter.** σ^S is the sigma factor responsible for the activation of many genes expressed mainly during the stationary phase (18). As shown above, transcriptional activation of the p1-*algD* promoter specifically occurs in the stationary phase. We cloned, as described in Materials and Methods, an *A. vinelandii rpoS* internal fragment encoding amino acids 60 to 313 of σ^S and constructed by reverse genetics strain CNS59, a derivative of ATCC 9046 carrying an *rpoS*::Sp mutation (see Materials and Methods). As predicted, transcription of *algD* from the p1 promoter in the CNS59 strain was not detected (Fig. 3), confirming that p1 is a σ^S-dependent promoter. In addition transcription from p2, the σ^E-dependent promoter during the stationary phase, was found to increase in the *rpoS* mutant (Fig. 3), suggesting that the absence of σ^S results in σ^E activation. Transcription from the p3 site was similar in the wild type and the *rpoS* mutant (data not shown). The *rpoS* mutation did not significantly affect the production of alginate (Table 2), suggesting that the increase in the activity of the p1 σ^E promoter compensates for the negative effect on the p2 σ^S promoter. These data suggest that both GacA and σ^S participate in the same regulatory cascade and that GacA functions upstream of σ^S.

Growth-phase-dependent expression of *rpoS* and its control by GacA. We determined the levels of *rpoS* mRNA by Northern analysis in cells of ATCC 9046 and the *gacA* mutant JM3 harvested from exponential (8 h) and stationary phase (48 h) cultures. In the wild-type strain ATCC 9046, *rpoS* mRNA was detected in the stationary phase but not during exponential growth (Fig. 4). Thus, as in other bacteria *rpoS* expression in *A. vinelandii* is under growth phase regulation. In *E. coli*, for example, the highest σ^S concentration is found in early stationary phase; however, a low-level expression of *rpoS* as determined by Northern blot analysis is detected in exponentially growing cells in minimal or rich media (23, 35). Correspondingly some σ^S-dependent genes are also expressed during exponential growth, implying a role for σ^S in growing cells (18). We did not detect *rpoS* RNA in exponential cultures grown in

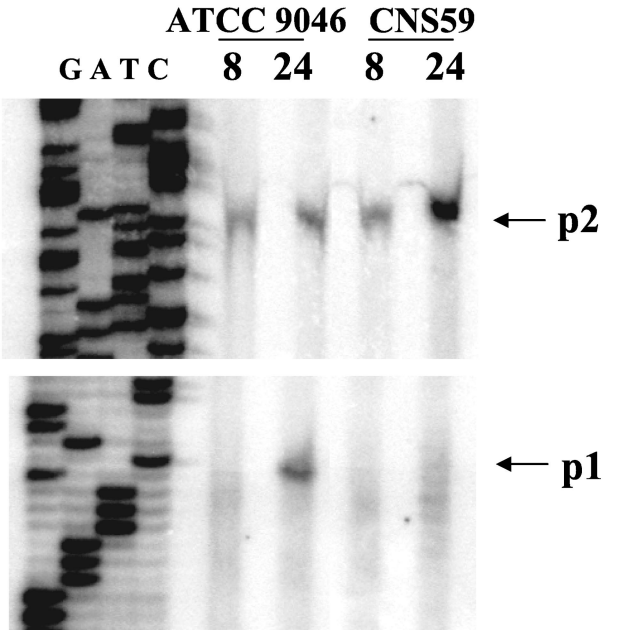

FIG. 3. Primer extension analysis of *algD* transcription from p1 and p2 in ATCC 9046 and CNS59 strains after 8 and 24 h of growth on Burk's sucrose medium.

Burk's minimal medium; however, as regulation of σ^S is unknown in *A. vinelandii*, this result does not rule out a role for this factor in growing cells.

The effect of the *gacA* mutation on transcription of the *rpoS* is also shown in Fig. 4. No RNA corresponding to *rpoS* was detected in the *gacA* mutant. Together, these results indicate that GacA mediates signal transduction between GacS and the activation of the *rpoS* promoter; in turn, σ^S mediates activation

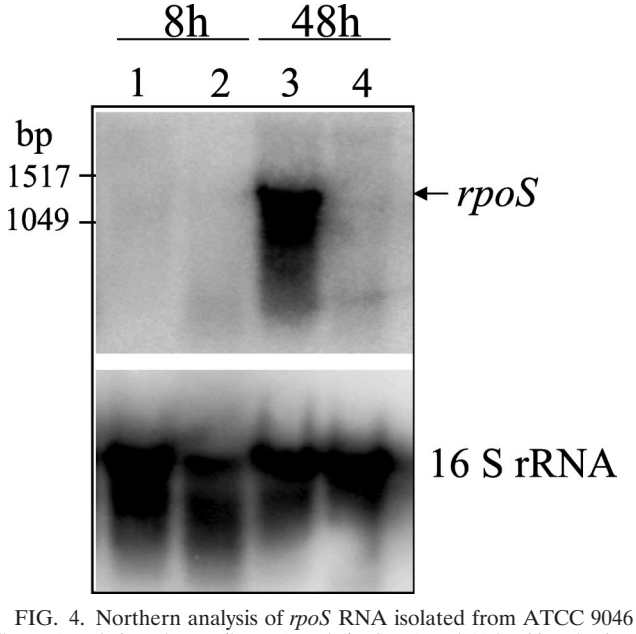

FIG. 4. Northern analysis of *rpoS* RNA isolated from ATCC 9046 (lanes 1 and 3) and JM3 (lanes 2 and 4) after 8 and 48 h of incubation in Burk's sucrose medium.

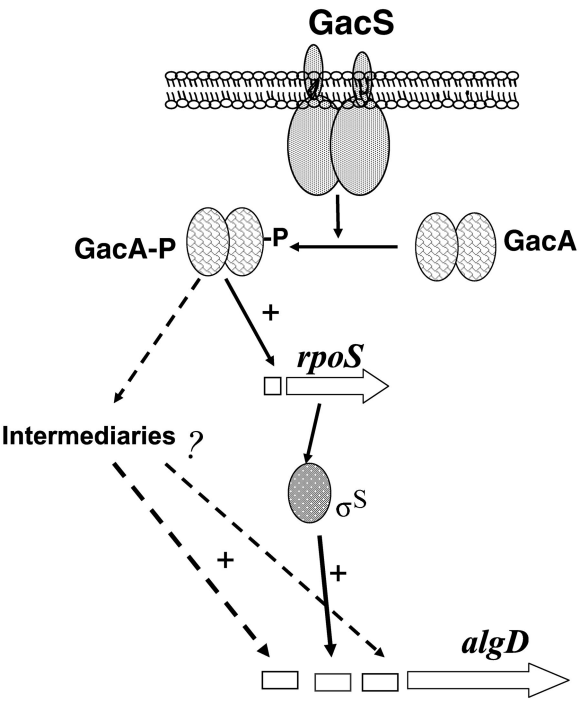

FIG. 5. Model for regulation of *algD* expression by the global regulators GacA and σ^S.

of the *algD*p1 promoter by GacA (Fig. 5). Whether GacA directly interacts with the *rpoS* promoter region remains to be determined.

The *gacA* mutation has no effect on *algU* transcription. The lack of transcription from p2 in JM3 during the stationary phase suggested that transcription of *algU*, the gene encoding σ^E, might be under GacA control. We carried out primer extension analysis of *algU*, with RNA isolated from strains ATCC 9046 and JM3 (data not shown). We found that the *gacA* mutation has no effect on transcription of *algU*; thus, stationary-phase induction of the *algD*-p2 promoter by GacA seems to be exerted via a σ^E-independent intermediary (Fig. 5).

The results of this study show that the *gacA* gene cloned encodes the cognate response regulator of GacS which is required for polymer synthesis and which is specifically required to activate transcription of *algD* from its three promoters, one of which was shown to be a σ^S-dependent promoter.

Activation of gene expression by the GacS/GacA system appears to use different signal pathways or cascades, one of which includes *rpoS*, since we showed that GacA is required for transcription of *rpoS*. By regulating expression of *rpoS*, the GacS/GacA system must play an important role in the control of stationary-phase functions. GacA was also shown to be required to activate the *algD* non-σ^S promoters; thus, activation of alginate synthesis by GacS/GacA is also mediated by another as-yet-unidentified pathway.

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