Azotobacter vinelandii Aldehyde Dehydrogenase Regulated by σ^{54}: Role in Alcohol Catabolism and Encystment

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Encystment in *Azotobacter vinelandii* is induced by *n*-butanol or β-hydroxybutyrate (BHB). We identified a gene, encoding an aldehyde dehydrogenase, that was named *aldA*. An *aldA* mutation impaired bacterial growth on *n*-butanol, ethanol, or hexanol as the sole carbon source. Expression of *aldA* increased in cells shifted from sucrose to *n*-butanol and was shown to be dependent on the alternative σ^{54} factor. A mutation in *rpoN* encoding the σ^{54} factor also impaired growth on alcohols. Encystment on *n*-butanol, but not on BHB, was impaired in *aldA* or *rpoN* mutants, indicating that *n*-butanol is not an inducer of encystment by itself but must be catabolized in order to induce encystment.

Azotobacter vinelandii is a soil nitrogen-fixing bacterium which produces the intracellular polyester poly-β-hydroxybutyrate (poly-BHB) and undergoes differentiation to form desiccation-resistant cysts. Encystment can be induced by removing sucrose from exponential-phase cells and replacing it with BHB or *n*-butanol (7, 20, 27): BHB, which is a natural metabolite in *A. vinelandii* (7), is believed to induce encystment because its addition results in conditions approximating poly-BHB degradation and because induction by *n*-butanol has been proposed to result from its conversion to BHB (20). Encystment induced by BHB or *n*-butanol or that occurring in late-stationary-phase glucose cultures is accompanied by the formation of a family of 5-*n*-alkylresorcinols and 6-*n*-alkylpirones that are lipids present only in encysting cells. They replace the membrane phospholipids and are a major component of the exine (16–18). Thus, encystment seems to be induced by a metabolic shift from carbohydrate metabolism to lipid metabolism, and this shift seems to be promoted in the presence of either *n*-butanol or BHB.

Aldehyde dehydrogenases participate in metabolic processes, such as the catabolism of alcohols (15); they catalyze the oxidation of aldehydes to their corresponding acid form. Some bacterial aldehyde dehydrogenases, such as *Pseudomonas aeruginosa* malonic semialdehyde dehydrogenase, have high specificity (12), while others such as acetaldehyde dehydrogenase II (AcDH-II) from *Ralstonia eutropha* function on a broad spectrum of substrates. AcDH-II is involved in the catabolism of acetoin as well as of ethanol (15).

We identified *aldA*, a gene encoding an aldehyde dehydrogenase, whose expression was increased in cells shifted from sucrose to *n*-butanol. We found that AldA activity is essential for catabolism of *n*-butanol and other alcohols and that *aldA*

expression is controlled by the alternative σ^{54} factor. We also show that *n*-butanol has to be catabolized to induce encystment.

MATERIALS AND METHODS

Microbiological procedures. The bacterial strains and plasmids used in this study are listed in Table 1. *A. vinelandii* was grown at 30°C on Burk's medium (5), supplemented with 2% sucrose, 0.2% *n*-butanol, or 0.2% BHB. *A. vinelandii* transformation was carried out as reported by Bali et al. (2). Encystment was determined by measuring resistance to desiccation as previously described (3). Bacterial strains were grown on liquid Burk's sucrose medium for 24 h, washed with 10 mM magnesium sulfate, and induced for encystment by incubation on plates of Burk's medium supplemented with *n*-butanol or BHB. β-Galactosidase activity was measured as reported by Miller (10); 1 U corresponds to 1 nmol of *o*-nitrophenyl-β-D-galactoside hydrolyzed per min per μg of protein. Protein was determined by the Lowry method (8). All measurements were done in triplicate.

Transposon mutagenesis. Random transposon mutagenesis of strain JG8 was carried out with a pUT derivative containing the mini-Tn5–*lacZ2* transposon as described previously (4). We isolated 3,200 kanamycin derivatives. Unexpectedly, all 3,200 mutants showed blue on plates containing Burk's sucrose medium with sucrose and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The parental strain JG8, used as a control, showed white. It is unlikely that, in all kanamycin-resistant mutants, the *lacZ* gene was fused to an *A. vinelandii* promoter, thus suggesting that the *lacZ* gene is transcribed in *A. vinelandii*. However, about 10% of the mutants were bluer, suggesting that, in these strains, expression of *lacZ* was also initiated from *A. vinelandii* promoters.

Construction of strains SG9A and CN10. Plasmid pSG9 (Fig. 1), which is unable to replicate in *A. vinelandii*, was introduced by transformation into strain ATCC 9046. Strain SG9A is a kanamycin-resistant derivative. The replacement of the *aldA* wild-type gene by the *aldA*–mini-Tn5 mutation on the chromosome of the SG9A was confirmed by Southern blotting using labeled pSG9 as a probe (data not shown).

A 2.3-kb *Cla*I-*Eco*RI restriction fragment from cosmid pLV72 (26) containing the *rpoN* gene from *A. vinelandii* was cloned into plasmid pBluescript KS, producing plasmid pSM6. A 0.8-kb *Kpn*I fragment containing a gentamicin (Gm) resistance gene from plasmid pBSL141 (1) was inserted in the *Kpn*I site within the *rpoN* gene present in pSM6 to create a *rpoN*::Gm mutation. The resultant plasmid pCN10, which is unable to replicate in *A. vinelandii*, was introduced by transformation into strain ATCC 9046. One gentamicin-resistant transformant strain named CN10 was chosen and confirmed by Southern analysis to carry the *rpoN*::Gm mutation (data not shown).

Recombinant DNA techniques. All DNA manipulations were performed by standard procedures (21).

Primer extension analysis. Primer extension reactions were performed with a primer extension system (Amersham) as instructed by the manufacturer. Oligo-

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
Strains (*A. vinelandii*)		
ATCC 9046	Highly mucoid	Our collection
JG8	*alg8*::Tc derivative of ATCC 9046	9
SG9	*aldA*::mini-Tn*5* derivative of JG8	This work
SG9A	*aldA*::mini-Tn*5* derivative of ATCC 9046	This work
SG9A::pSM9-Gm	derivative of SG9A with pSM9-Gm integrated in the chromosome	This work
CN10	*rpoN*::Gm derivative of ATCC 9046	This work
Plasmids		
pBluescript SK(+)	Plasmid used for subcloning DNA, Ap^r	Stratagene
pUT-mini-Tn*5*–*lacZ*	Suicide vector for mutagenesis with mini-Tn*5*–*lacZ*	4
pBSL141		1
pSM888	Derivative of pCP13 carrying *A. vinelandii aldA* region	This work
pSG9	Derivative of pBluescript SK(+) carrying the *aldA*::Tn*5* mutation from SG9	This work
pSM9	Derivative of pBluescript SK(+) carrying the *aldA* wild-type gene	This work
pSM9-Gm	Derivative of pSM9 with a Gm resistance gene	This work
pLV72	Cosmid clone with the *A. vinelandii rpoN* gene	26
pSM6	Derivative of pBluescript SK(+) carrying the *A. vinelandii rpoN* gene in a 2.3-kb *Eco*RI-*Cla*I fragment	This work
pCN10	pSM6 derivative carrying a *rpoN*::Gm mutation	This work

nucleotide primer 5′CACTGCCAGGATGGGCATAC was labeled with [γ-^{32}P]dATP (Amersham) at the 5′ end by using polynucleotide kinase and was 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 pSG9 DNA by the dideoxy chain method (22), using [γ-^{32}P]dATP and a sequencing kit with the same primer employed for the primer extension reactions.

Aldehyde dehydrogenase assay. *A. vinelandii* cells were grown to early stationary phase on Burk's sucrose medium at 30°C. The cultures were centrifuged, washed, and resuspended in Burk's *n*-butanol medium. After 20 h of induction in this medium, the cells were collected by centrifugation, resuspended in 1/25 volume of phosphate buffer, pH 7.4, and sonicated. The sonicated cell suspensions were centrifuged at 14,000 × *g* for 5 min, and the supernatants were centrifuged for 1.5 h at 192,000 × *g*. Ammonium sulfate was added to give 50% saturation. The precipitate was collected by centrifugation at 14,000 × *g*, resuspended in phosphate buffer, pH 7.4, and dialyzed against the same buffer. The activity was assayed spectrophotometrically as reported previously (13) by measuring the reduction of NAD$^+$ at 340 nm. The reaction was initiated by addition of butyraldehyde or acetaldehyde. The rate of endogenous NAD$^+$ reduction was recorded in the absence of butyraldehyde or acetaldehyde in the assay. One unit of activity is defined as the amount of enzyme reducing 1 nmol of NAD$^+$ per min per mg of protein.

Cloning of *aldA* gene. A cosmid clone pSM888 from an *A. vinelandii* gene library that harbored a 2.2-kb *Pst*I fragment containing the *aldA* gene was identified by hybridization with a 0.5-kb *Sal*I fragment from pSG9. The 2.2-kb *Pst*I fragment was subcloned into plasmid pKS to give plasmid pSM9 (Fig. 1). A Gm resistance gene was cloned into the polylinker of pSM9 to give plasmid pSM9-Gm (Fig. 1).

Nucleotide sequence accession number. The *A. vinelandii aldA* gene sequence reported here has been assigned GenBank accession no. AF277380.

RESULTS

Identification of *A. vinelandii* gene induced in *n*-butanol. In *A. vinelandii*, encystment can be induced by transferring vegetative cells into plates of Burk's salt medium containing sublethal levels (0.2%) of *n*-butanol as the carbon source. To identify genes expressed during the induction of the encystment process, we isolated 3,200 mutants carrying mini-Tn*5*–*lacZ* insertions (see Materials and Methods). When mini-Tn*5*–*lacZ* is inserted downstream, a promoter in the correct orientation creates a gene fusion. We transferred the 3,200 mini-Tn*5*–*lacZ* mutants to plates of Burk's medium containing *n*-butanol as the only carbon source and X-Gal and to plates with Burk's medium with 2.0% sucrose and X-Gal. A mutant named SG9, that, although unable to grow on Burk's butanol, showed a bluer phenotype on Burk's butanol than on Burk's sucrose, was identified (data not shown). A 7.0-kb *Pst*I fragment containing the mini-Tn*5*–*lacZ* insertion from SG9 was cloned into pBluescript KS. The resultant plasmid pSG9 (Fig. 1) was transformed to wild-type ATCC 9046. Strain SG9A, a
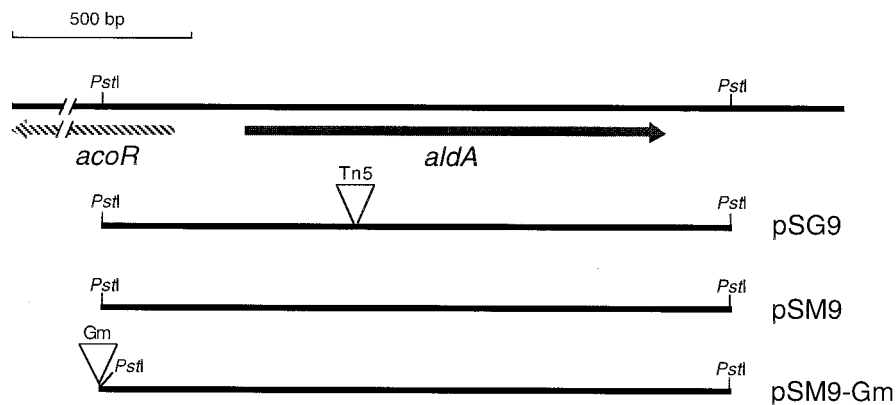

FIG. 1. Physical map of the *A. vinelandii aldA-acoR* chromosomal region and plasmids carrying this region.
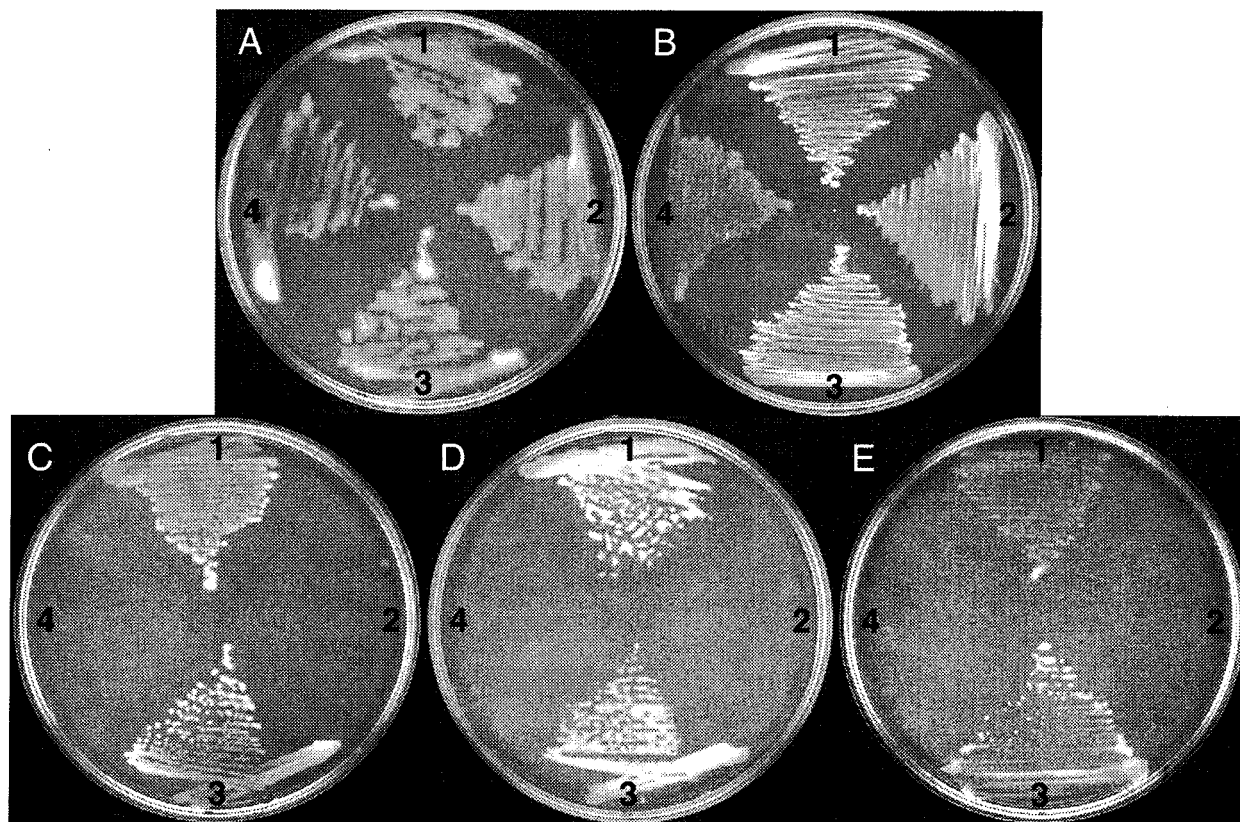
FIG. 2. Growth phenotypes of *A. vinelandii* ATCC 9046 (1), SG9A (2), SG9A::pSM9-Gm (3), and CN10 (4) at 30°C on Burk's medium supplemented with 10 mM NH₄Cl as the nitrogen source and with 2.0% sucrose (A), 0.2% BHB (B), *n*-butanol (C), ethanol (D), or hexanol (E) as the sole carbon source.

transformant that was unable to grow on Burk's butanol (Fig. 2C) and showed a bluer phenotype on Burk's butanol was isolated, confirming that these phenotypes were caused by the mini-Tn*5* insertion. We also confirmed induction of transcription under encystment conditions of the identified gene by measuring β-galactosidase activity. Cultures of strain SG9A were grown for 24 h in liquid Burk's medium supplemented with 2.0% sucrose. Cells from these cultures were transferred to agar plates containing Burk's medium supplemented with *n*-butanol or sucrose. As expected, an increase in β-galactosidase activity was observed during incubation on *n*-butanol (Fig. 3).

Cloning and DNA sequence. Plasmid pSG9 (Fig. 1) was used to determine 2.3 kb of the *A. vinelandii* DNA sequence surrounding the transposon. Analysis of this sequence revealed an open reading frame encoding a polypeptide of 506 amino acid residues with a calculated molecular mass of 55,061 Da. The location of the mini-Tn*5–lacZ* mutation was determined by sequencing across the transposon insertion junction and was found to lie within codons 135 to 137. Comparison of the deduced amino acid sequence with those in the databases revealed a high degree of similarity with aldehyde dehydrogenases from different sources. Thus, we named this *A. vinelandii* gene *aldA*. The deduced protein product shares 89% identity with the *P. aeruginosa* putative aldehyde dehydrogenase PA4022 (24) (accession no. F83142), 88% with *P. aeruginosa* ExaC (23) (accession no. AF068264), and 69% with *R. eutropha* AcDH-II (P46368), an enzyme responsible for the oxidation of acetal-

dehyde to acetate during growth on ethanol or acetoin (15). In the sequenced DNA fragment, 303 nucleotides upstream of *aldA*, an incomplete open reading frame comprised of 207 nucleotides and divergently oriented is present (Fig. 1). A Blast P GenBank search for the deduced protein revealed similarity to the acetoin catabolism regulatory protein AcoR (SWISS PROT P28614), the putative activator for acetoin catabolism genes of *R. eutropha* H16 (6). It is a member of the NifA family of transcriptional activators known as enhancer binding proteins, required for transcription from σ⁵⁴-dependent genes (11). The highest identity of this *A. vinelandii* AcoR homologue (89%) was found with the *P. aeruginosa* PAO1 putative transcriptional activator PA4021, which is located upstream and in opposite orientation to that of the putative aldehyde dehydrogenase PA4022 (24).

Effect of *aldA* mutation on aldehyde dehydrogenase activity. The inability of SG9A to grow on the carbon source *n*-butanol and the high similarity of *aldA* with aldehyde dehydrogenases suggested that this gene encodes an enzyme with butyraldehyde dehydrogenase activity. We assayed this activity in extracts of the wild-type strain ATCC 9046 and SG9A.

After induction on *n*-butanol, coenzyme A-independent butyraldehyde dehydrogenase activity was detected in the wild type and a significant reduction of this activity was found in the SG9A mutant (Table 2). The *aldA* mutation did not completely abrogate butyraldehyde activity, suggesting that another enzyme is the source of the background activity. AldA activity on
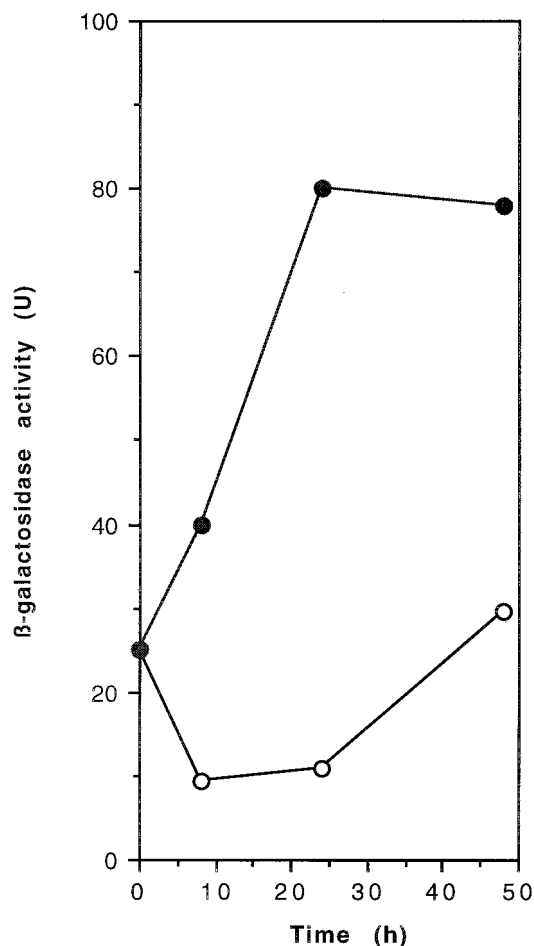
FIG. 3. β-Galactosidase activity of strain SG9A, during incubation in solid Burk's medium with sucrose (open symbols) or *n*-butanol (closed symbols).
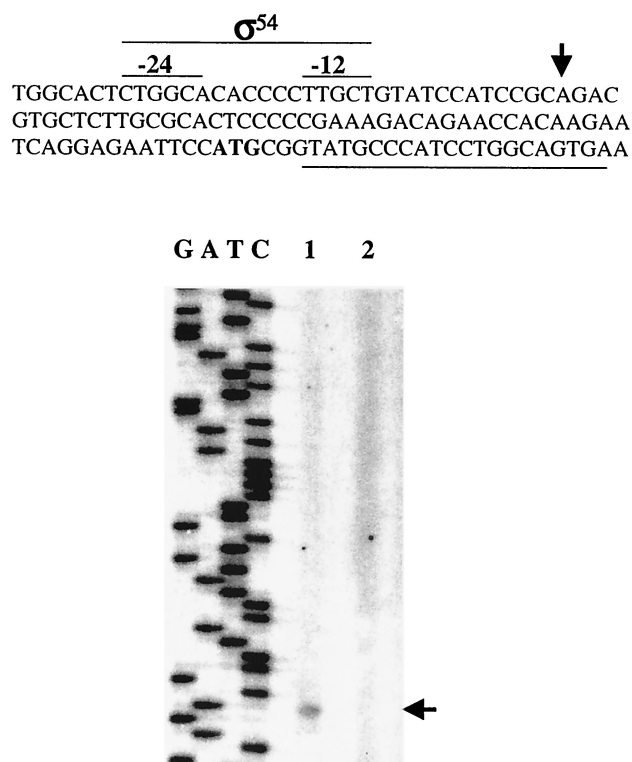

FIG. 4. Primer extension analysis of *aldA* transcription in strains ATCC 9046 and CN10. (A) DNA sequence of the 5′ end of *aldA*. The arrow indicates the start site of *aldA* transcription; the σ^{54} promoter is also indicated at the top. The ATG initiation codon is shown in bold-face. The complementary sequence where the oligonucleotide used for primer extension was generated is underlined. (B) Primer extension of the *aldA* gene in strain ATCC 9046 (lane 1) and in strain CN10 (lane 2) incubated in Burk's medium plus ammonia with sucrose. The *aldA* sequence ladder (GATC) was produced with the oligonucleotide used for primer extension.

acetaldehyde as a substrate was also tested. As shown in Table 2, AldA also displays acetaldehyde dehydrogenase activity. In agreement with this result, strain SG9A was unable to grow with ethanol or hexanol as the carbon source (Fig. 2D and E). These data indicate that the AldA activity is not specific for *n*-butanol and participates in the catabolism of several alcohols.

Identification of an *aldA* σ^{54} promoter. Primer extension experiments were performed to determine the transcriptional start site of *aldA*. Figure 4 shows the primer extension products obtained with RNA isolated from strain ATCC 9046 grown in Burk's medium supplemented with *n*-butanol. A transcrip-

tional start site corresponding to an A residue located 57 nucleotides upstream of the proposed *aldA* start codon was identified (Fig. 4). Examination of the 5′ upstream region revealed the presence of putative −24 (CTGGCA) and −12 (TTGCT) promoter sequences recognized by σ^{54}. We therefore carried out primer extension analysis of *aldA* in strain CN10, an ATCC 9046 derivative carrying a mutation in *rpoN* encoding σ^{54}. As shown in Fig. 4, no primer extension products were detected with RNA from strain CN10.

Effect of *rpoN* mutation on aldehyde dehydrogenase activity and on growth on alcohols. As transcription of *aldA* seems to be impaired by the *rpoN* mutation, growth on alcohols and aldehyde dehydrogenase activity in the *rpoN* mutant were expected to be similar to those of the *aldA* mutant. The results shown in Table 2 and Fig. 2 indicate that this is indeed the case and confirm the dependence of the *aldA* promoter on the σ^{54} factor. Together these data confirm that *aldA* is transcribed from a single σ^{54} promoter and that its product AldA is essential for the utilization of several alcohols as carbon sources.

Effect of *aldA* and *rpoN* mutations on encystment. Induction of encystment by *n*-butanol is proposed to be due to its conversion to BHB (20). If this hypothesis is correct, AldA and σ^{54} are expected to be essential for encystment induced by *n*-butanol. We tested encystment of strains SG9A and CN10 in

TABLE 2. Aldehyde dehydrogenase activity in
A. vinelandii aldA mutant[a]

Strain	Genotype	Activity of aldehyde dehydrogenase (nmol min^{-1} mg^{-1})[b] found with:	
		Butyraldehyde	Acetaldehyde
ATCC 9046	Wild type	75.0 ± 3.0	104.1 ± 3.0
SG9A	*aldA*	20.4 ± 1.4	23.6 ± 2.6
CN10	*rpoN*	22.4 ± 1.7	21.3 ± 0.6

[a] Cells were induced on Burk's medium with *n*-butanol as the sole carbon source.
[b] Values are the mean of three determinations ± standard deviations.

TABLE 3. Encystment in *A. vinelandii aldA* and *rpoN* mutants

Strain	Resistance to desiccation (%)[a] with:	
	n-Butanol	BHB
ATCC 9046	17.9 ± 4.0	11.6 ± 2.4
SG9A	$<10^{-4}$	1.3 ± 0.03
SG9A::pSM9-Gm	13 ± 3.4	ND
CN10	$<10^{-4}$	10.0 ± 2.8

[a] Resistance to desiccation of cells induced on Burk's medium with *n*-butanol or BHB as the sole carbon source. Values are the mean of three determinations. Values are given $\pm$ standard deviations. ND, not determined.

either *n*-butanol or BHB. As expected, strain SG9A was unable to produce desiccation-resistant cells when induced with *n*-butanol but not with BHB (Table 3). Growth of strain SG9A on BHB is similar to that of the wild-type strain (Fig. 2). However, it produced cysts resistant to desiccation with a reduced frequency when BHB rather than *n*-butanol was the sole carbon source (Table 3). This result supports the hypothesis that *n*-butanol induces encystment because it is converted to BHB; however, it does not rule out the possibility that the lack of encystment of SG9A is caused by its inability to grow on *n*-butanol. We added 0.1% sucrose to the Burk's *n*-butanol encysting medium to promote growth of strain SG9A during encystment; however, no mature cysts were detected under this condition, either in the wild type or the *aldA* mutant (data not shown). This result is in agreement with the abortion of encystment that was observed when glucose was added to encysting *A. vinelandii* cultures (7).

We tested encystment of strain CN10. Similar to strain SG9A, strain CN10 formed mature cysts when induced with BHB but not with *n*-butanol (Table 3). As the *rpoN* mutation impairs diazotrophic growth (26), this result implies that encystment by strain CN10 in Burk's medium supplemented with BHB in the absence of ammonia occurred under nongrowing conditions. These data further suggest that the inability to catabolize *n*-butanol imposed by the *aldA* or the *rpoN* mutation causes its cyst-defective phenotype and support the hypothesis that catabolism of *n*-butanol is essential to induce encystment.

SG9A complementation by *aldA* wild-type gene. Plasmid pSM9-Gm (Fig. 1), which is unable to replicate in *A. vinelandii* and carries the *aldA* gene flanked by 500 nucleotides upstream of the ATG start codon and 200 nucleotides downstream of the stop codon, was transformed into SG9A for integration into the chromosome. Strain SG9A::pSM9-Gm, a gentamicin-resistant transformant, was selected. The presence of a wild-type *aldA* gene was confirmed by PCR analysis using SG9A::pSM9-Gm chromosomal DNA as a template as well as oligonucleotides aldA1 5'- and aldA2 3'- (data not shown). Strain SG9A::pSM9-Gm grew in *n*-butanol, ethanol, or hexanol (Fig. 2) and produced cells resistant to desiccation after induction with *n*-butanol (Table 3). We do not know whether the *aldA* mutation has a polar effect on downstream genes; however, integration of pSM9-Gm downstream of the mini-Tn5–*lacZ* insertion (in strain SG9A::pSM9-Gm) should restore the activity of these genes. Therefore, this result confirms that, either polar or nonpolar, the *aldA* mutation causes an inability to grow on alcohols and to encyst.

DISCUSSION

In an effort to understand the nature of genes involved in the induction of the encystment and the molecular mechanisms that control this process in *A. vinelandii*, we have identified, through mini-Tn5–*lacZ* mutagenesis and subsequent screening for *lacZ* gene fusions whose expression increased when shifted from sucrose to *n*-butanol, a gene encoding a protein with high similarity to aldehyde dehydrogenases. The gene was named *aldA*. Inactivation of *aldA* by mini-Tn5–*lacZ* led to a significant reduction in aldehyde dehydrogenase activity, demonstrating that this gene indeed encodes an aldehyde dehydrogenase. Inactivation of *aldA* also led to the inability to grow on *n*-butanol, ethanol, or hexanol as the sole carbon source. Thus, AldA is a wide substrate specificity enzyme that is responsible for the oxidation of aldehydes during growth on alcohols.

An interesting finding was the presence upstream from and in opposite orientation to *aldA* of an open reading frame whose deduced amino acid sequence is highly similar to that of the activator AcoR. The highest similarity of both AldA and this AcoR homologue was found with the not-yet-characterized *P. aeruginosa* PA4022 and PA4021 gene products, respectively. The physical organization of these two genes was also similar in both bacteria. It would be interesting to find out whether these *P. aeruginosa* genes are involved in alcohol catabolism.

This study demonstrates that expression of *aldA* is under σ^{54} control. σ^{54} controls diverse and unrelated functions in different groups of bacteria (for a recent review, see reference 25). Some of these functions include the catabolism of acetoin and ethanol in *Alcaligenes eutrophus* (13, 14). Our finding that *aldA* is transcribed from a σ^{54}-dependent promoter and the inability of *rpoN* and *aldA* mutants to grow on ethanol, *n*-butanol, or hexanol add the catabolism of these alcohols to the processes controlled by *rpoN*.

Transcription from σ^{54}-dependent genes requires additional transcriptional factors known as enhancer binding proteins (11); this implies the requirement of an activator protein for *aldA* transcription. In fact the presence upstream of *aldA* of an *acoR* homologue, encoding a member of the NifA family of transcriptional activators, suggests that this AcoR might be the activator of the *aldA* promoter. In *R. eutropha*, AcoR is the putative activator for acetoin and AcDH-II genes (6).

The inability of *rpoN* and *aldA* mutants to form mature cysts on *n*-butanol but not in BHB supports the hypothesis that *n*-butanol is not by itself an inductor but that it induces encystment because it is converted to BHB or other related metabolites. The lack of encystment by SG9A and CN10 on *n*-butanol could be caused by their inability to grow under these conditions. However, we showed that encystment can occur under nongrowing conditions, as was the case for strain CN10 when it was induced in BHB without ammonia. In addition, encystment is initiated by the rounding up of the vegetative cell and the termination of cell division (19).

It is important that lack of growth of strain CN10 on BHB in the absence of ammonia is due to the negative effect of the *rpoN* mutation on nitrogen fixation and that this process has been shown to be arrested upon initiation of encystment (19, 20). In contrast, lack of growth on *n*-butanol of either SG9A or CN10 strain is caused by each strain's inability to catabolize

alcohols. Thus, a source of carbon (able to induce a shift from glucose to BHB metabolism) seems to be essential for the encystment process even under nongrowing conditions.

In conclusion, we identified an aldehyde dehydrogenase AldA gene regulated by σ^{54} that is essential for the catabolism of alcohols and provided evidence that the catabolism of *n*-butanol by AldA activity is required to induce encystment.

ACKNOWLEDGMENTS

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