Preliminary Characterization of BYN4, a *Rhodobacter sphaeroides* Mutant Affected in Alcohol Metabolism

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ABSTRACT

Rhodobacter sphaeroides is a Gram-negative, purple photosynthetic bacterium capable of using several alcohols as carbon and energy sources. Previous study of mutants unable to utilize alcohols has demonstrated that the co-enzyme PQQ is essential for butanol and methanol metabolism. The purpose of this study was to identify and characterize additional genes involved in alcohol metabolism. Previous research identified four transposon mutants unable to metabolize 3-butyn-1-ol, a suicide substrate for alcohol dehydrogenase enzymes. Complementation tests showed that genes involved in PQQ biosynthesis were unable to restore a wild-type phenotype to two of these mutants, BYN2 and BYN4. In this work, a 9.5 kb *Bam*HI fragment of BYN4, containing the transposon and flanking *R. sphaeroides* DNA, was cloned and used to probe a cosmid library of *R. sphaeroides* DNA. Hybridizations indicated that the probe hybridized to an approximately 4 kb *Bam*HI fragment of cosmid 473. Complementation studies with cosmid 473 and an overlapping cosmid, 747, showed that the wild-type DNA contained in each of these cosmids restores the ability of BYN4 to metabolize 3-butyn-1-ol. Preliminary sequence data indicates that the gene(s) mutated in BYN4 may encode a protein involved in a two-component regulatory system responsible for transcriptional regulation of genes essential for alcohol metabolism.

INTRODUCTION

Rhodobacter sphaeroides is a Gram-negative photosynthetic bacterium that boasts an extremely diverse metabolism. This bacterium can grow aerobically, anaerobically by photosynthesis, and anaerobically in the dark when provided with an external terminal electron acceptor such as dimethyl sulfoxide (DMSO), trimethylamine N-oxide (TMAO), or nitrous oxide (4). *R. sphaeroides* is also able to utilize some alcohols as carbon and energy sources.

To metabolize alcohols, bacteria use internal enzymes known as alcohol dehydrogenases (ADHs) to break down the alcohol into compounds that can be readily incorporated into cellular material. From its ability to grow on alcohols such as methanol, butanol, and isobutanol (7), it is clear that *R. sphaeroides* contains at least one ADH enzyme. Many ADHs require enzyme cofactors such as NADP+ or pyrrolo quinoline-quinone (PQQ). Previous research has shown that PQQ is essential for methanol and butanol

metabolism in R. *sphaeroides* (8).

The goal of this study was to identify and characterize additional genes involved in alcohol metabolism in *R. sphaeroides*, other than those for PQQ biosynthesis. Transposon mutagenesis using Tn5Tp (12) yielded four mutants resistant to 3-butyn-1-ol, a suicide substrate for functional ADH enzymes which normally oxidize 3-butyn-1-ol to a toxic aldehyde. Genes for PQQ biosynthesis were introduced in trans into each of the mutants and were unable to restore 3-butyn-1-ol sensitivity to two of these mutants, BYN2 and BYN4. Clones pBS2 and pBS4 contain the Tn5-interrupted region of DNA from BYN2 and BYN4 in pGEM-3zf(+), respectively (15). This paper presents preliminary evidence that the gene(s) mutated in BYN4 may be involved in a two-component regulatory system responsible for transcriptional regulation of genes essential for alcohol metabolism in *R. sphaeroides*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study are listed in Table 1. *R. sphaeroides* strains were cultured in Sistrom's minimal medium A (SIS) at 32°C (14). Trimethoprim (Tp), suspended in N, N-dimethylformamide, was added to a concentration of 30 µg/ml for strains BYN2 and BYN4. *E. coli* strains were grown in Luria Bertani (LB) medium at 37°C. Tetracycline (Tc) was used in a concentration of 1 µg/ml for *R. sphaeroides* and 5 µg/ml for *E. coli* to maintain cosmids containing *R. sphaeroides* chromosomal DNA (5). The concentration of Tc used to maintain pMAR1 in *E. coli* was increased to 10 µg/ml. For transformations, ampicillin (Ap) was added to LB medium at 50 µg/ml to maintain pGEM-3zf(+)-derived plasmids in *E. coli* and X-gal (5-bromo-4-chloro-3-indoyl-ß-D-galactoside) was added to 40 µg/ml to screen for non-ß-galactosidase-producing colonies.

In complementation studies, cell growth was measured turbidometrically (19) using a Klett Summerson colorimeter equipped with a No. 66 filter (Carolina Biological Supply, Burlington, NC). One Klett unit was assumed to equal 1 x 10^7 cells/ml. *R. sphaeroides* strains receiving cosmid DNA were patched onto SIS + 3-butyn-1-ol (0.4%) + Tc and onto SIS + Tc.

DNA manipulations. Restriction mapping of pBS2 and pBS4 was performed according to Sambrook (11). Restriction enzymes were purchased from New England Biolabs (Beverly, MA), Promega Corp. (Madison, WI) or Gibco BRL (Gaithersburg, MD) and were used according to the manufacturer's specifications. Minipreparation of plasmid and cosmid DNA was accomplished with the alkaline sodium dodecyl sulfate lysis method of Sambrook (11). All DNA fragments used for probe construction and subcloning were purified from 0.8% agarose gels using the Geneclean II®(kit (Bio 101, San Diego, CA).

Hybridization techniques. The 9.5 kb *Bam*HI fragment of pBS4 was purified and radiolabeled with [α^{32}P]-dGTP using the Nick Translation System of Gibco BRL (Gaithersburg, MD). Southern blots containing *Bam*HI digests of groups of five *E. coli* cosmid strains harboring *R. sphaeroides* chromosomal DNA were then probed (5,8). Membranes were washed under the following stringency conditions: 1X SSC, 0.1% SDS, 42° C for 20 min and 0.1X SSC, 0.1% SDS, 42° C for 20 min. Following overnight exposure, film was fully processed.

TABLE 1. Bacterial strains, plasmids, and cosmids.

Strain or plasmid	Description	Source or Reference
R. sphaeroides		
2.4.1.	Wild-type	Laboratory Strain
BYN2	Wild-type parent, Tn5Tp^r, 3-butyn-1-ol^r	15
BYN4	Wild-type parent, Tn5Tp^r, 3-butyn-1-ol^r	15
E. coli		
DH5α	Transformation strain, *rec*A1	3
S17-1A	Cosmid-harboring strain, conjugable with *R. sphaeroides*	13
Plasmids		
pGEM-3zf(+)	Ap^r, pUC derivative	9
pLA2917	Tc^r, cosmid parent plasmid	1
pMAR1	2.5 kb *Bam*HI-*Hind*III fragment containing *cycI* and *adhI* from cosmid 747 cloned into pRK415	2,10
pRK415	Tc^r, RK2 derivative	6
pLD11	4.0 kb *Bam*HI-*Eco*RI fragment from pBS4 cloned into pGEM-3zf(+)	This work
pBS2	9.5 kb *Bam*HI fragment containing Tn5 from BYN2 cloned into pGEM-3zf(+)	15
pBS4	9.5 kb *Bam*HI fragment containing Tn5 from BYN4 cloned into pGEM-3zf(+)	15
Cosmids		
cosmid 473	Tc^r *byn-4* DNA in pLA2917	5
cosmid 747	Tc^r *byn-4* DNA in pLA2917	5
cosmids 471-475	Tc^r *R. sphaeroides* DNA in pLA2917	5
cosmids 186-190	Tc^r *R. sphaeroides* DNA in pLA2917	5

Construction of strains and complementation testing. Cosmids 186-190, 471-475, 747 and plasmids pMAR1 and pLA2917 were mobilized from *E. coli* S17-1A to BYN2 and BYN4 as needed according to the following procedure. Exactly 1.5 ml of a saturated *E. coli* donor was harvested for 20 s at 14,000 rpm in a microcentrifuge and the supernatant was discarded. On top of this pellet, 600 µl of the *R. sphaeroides* recipient at a cell density of approximately 150 KU/ml was harvested and the supernatant discarded again. Cells were resuspended in 150 µl of SIS with gentle vortexing and 100 µl of the resuspension was spread onto an LB plate and incubated for 1 hour at 32° C to allow conjugal transfer. Cells were then replica plated onto SIS containing Tc. After 3-4 days of growth about 50 colonies were patched first onto SIS + Tc + 3-butyn-1-ol and immediately onto SIS + Tc.

Subcloning strategy. A 4.1 kb *Bam*HI-*Eco*RI fragment of pBS4 was purified and ligations with *Bam*HI-*Eco*RI digested pGEM-3zf(+) were set up at an insert to vector molar ratio of 3:1 according to Sambrook (11). Competent *E. coli* DH5α cells were prepared (3), and transformations were conducted according to the procedure of Sambrook (11). White colonies were cultured in LB + Ap and miniprepped after overnight growth. Miniprep plasmid DNA was then digested with *Bam*HI and *Eco*RI and electrophoresed alongside a *Bam*HI-*Eco*RI digest of pBS4 to identify the desired clone.

DNA sequencing and analysis. The IS50 Tn5 primer 5' -ACGTTACCATGTTAGG-3' was used to sequence out of the transposon DNA and into the flanking *R. sphaeroides* DNA contained within pLD11. A synthetic primer 5' -AACTGGTGGGGCTCTTATTC-3' was constructed by Genosys Corp. (The Woodlands, TX) and used in a second sequencing run. All sequencing reactions were performed at the Biotechnology Center at

the University of Wisconsin-Madison and sequence data was analyzed at the amino acid level with the BLITZ program (16,18).

RESULTS

Restriction pattern of pBS2 and pBS4. Both pBS2 and pBS4 are clones of the *R. sphaeroides* *Bam*HI fragment containing the transposon in BYN2 and BYN4, respectively (15). The insertion sequences in Tn*5* contain a *Hpa*I site and the transposon contains a single *Eco*RI site. In order to facilitate further studies, a preliminary pattern of restriction enzyme sites within pBS2 and pBS4 was determined using the restriction enzymes *Bam*HI, *Hpa*I and *Eco*RI.

Results of *Bam*HI and *Bam*HI-*Hpa*I digests of pBS2 and pBS4 are shown in Fig. 1. *Bam*HI digests of both pBS2 and pBS4 result in fragments of 9.5 kb and 3.2 kb. Other fragments present in these digests (of greater number with pBS2) may be due to incomplete digestion during the original cloning procedure but are insignificant to this study. The 3.2 kb fragment corresponds to the cloning vector, pGEM-3zf(+), and the 9.5 kb fragment contains the transposon (5.5 kb) and flanking *R. sphaeroides* chromosomal DNA (~4 kb). Upon double digestion of pBS2 with *Bam*HI and *Hpa*I, the 3.2 kb *Bam*HI vector fragment remains and the 9.5 kb *Bam*HI fragment disappears. A new 5.5 kb *Hpa*I fragment (Tn5) and two *Bam*HI-*Hpa*I *R. sphaeroides* DNA fragments of 3.3 kb and 1.6 result.

Upon double digestion of pBS4 with *Bam*HI and *Hpa*I, again the 3.2 kb vector band remains. The 9.5 kb *Bam*HI fragment is reduced to a 5.5 kb *Hpa*I fragment (Tn5) and two 2.3 kb *Bam*HI-HpaI fragments show as a doublet on the gel. This doublet suggests that the transposon in pBS4 is flanked by 2.3 kb of *R. sphaeroides* DNA on each side. Due to the smaller number of bands present in digests of pBS4, this clone was chosen for further study and is the focus of the majority of this paper.

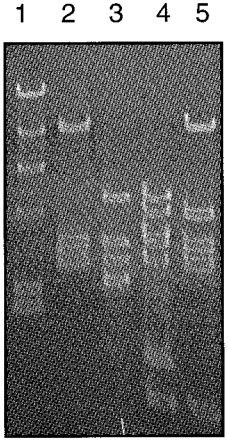

FIG. 1. Results of *Bam*HI and *Bam*HI-*Hpa*I digests of pBS2 and pBS4 DNA separated on a 0.8% agarose gel. Lane 1 contains a *Hind*III digest of λDNA, used as a size standard. Lanes 2 and 3 contain *Bam*HI and *Bam*HI-*Hpa*I digests of pBS4, respectively. Lanes 4 and 5 represent *Bam*HI-*Hpa*I and *Bam*HI digests of pBS2, respectively.

In order to determine the location of the *Eco*RI site within the transposon, a *Bam*HI-*Eco*RI digest of pBS4 was prepared (see Fig. 2). The 9.5 kb *Bam*HI fragment was replaced by *Bam*HI-*Eco*RI fragments of 5.6 kb and 4.1 kb. Knowing that the transposon is flanked by 2.3 kb of *R. sphaeroides* DNA on each side, we determined that the *Eco*RI site lies approximately 1.8 kb in from one end of the transposon. A general diagram of the pBS4 construction is shown in Fig. 3.

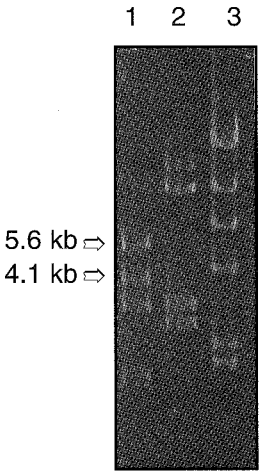

FIG. 2. Comparison of *Bam*HI and *Bam*HI-*Eco*RI digests of pBS4 DNA run on a 0.8% agarose gel. Lanes 1 and 2 contain *Bam*HI and *Bam*HI-*Eco*RI digests of pBS4, respectively. Lane 3 contains a *Hind*III digest of λDNA used as a size standard.

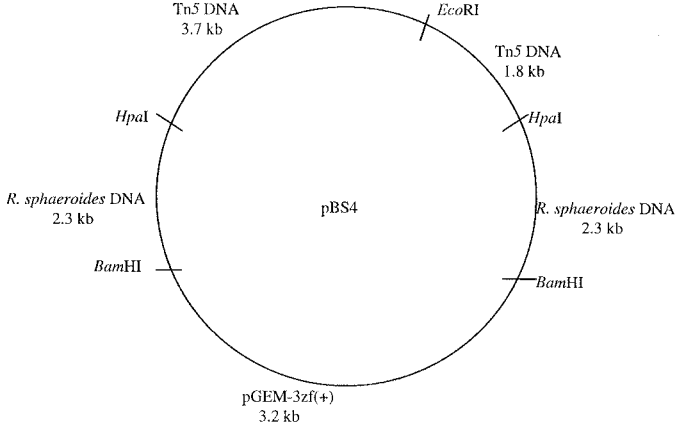

FIG. 3. The construction of pBS4, showing *Bam*HI, HpaI, and *Eco*RI sites and regions of vector, transposon, and *R. sphaeroides* DNA.

BYN4 DNA hybridizes to cosmid 473. The entire 9.5 kb *Bam*HI fragment of pBS4 was used to probe a cosmid library of wild-type *R. sphaeroides* DNA. Initial results showed hybridization to an approximately 4 kb *Bam*HI fragment of *R. sphaeroides* DNA contained in the lanes representing cosmids 471-475 and 186-190 (Fig. 4). Each of these cosmids were examined individually and hybridization was seen only with cosmid 473 (data not shown). Contrary to what we expected, no hybridization was seen with individual cosmids 186-190. Gel photographs of the first Southern blots probed were unavailable, so the original group of cosmids showing hybridization may have been a group other than 186-190.

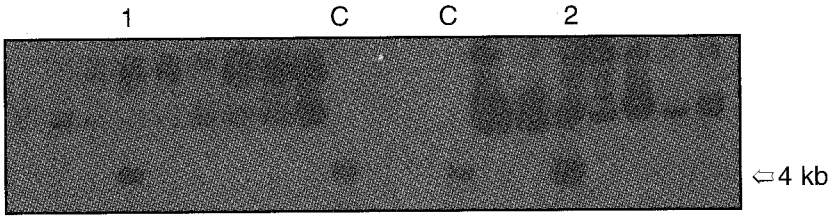

FIG. 4. Groups of 5 cosmid strains harboring *R. sphaeroides* chromosomal DNA were probed with the [α^{32}P]-dGTP labeled 9.5 kb *Bam*HI fragment of pBS4. The control lanes (C) contain *Bam*HI digests of wild-type R. sphaeroides genomic DNA and hybridization is seen to an approximately 4 kb *Bam*HI fragment. Hybridization of a similar sized fragment is also seen in the lanes representing cosmids 471-475 (lane 1) and 186-190 (lane 2).

Cosmids 473 and 747 complement the phenotype of BYN2 and BYN4. Previous research has shown that cosmid 473 and cosmid 747 contain some overlapping *R. sphaeroides* DNA (10). Since BYN4 DNA hybridized to cosmid 473 DNA, we wished to determine whether the DNA in cosmids 473 and/or 747 could restore a wild-type phenotype (3-butyn-1-ol sensitivity) to BYN2 and BYN4. These cosmids were separately mobilized into BYN2 and BYN4 and resultant strains were tested for their ability to grow in the presence of 3-butyn-1-ol. Both cosmids were able to restore a wild-type phenotype to each of the mutants (Table 2). The parent plasmid for the cosmid strains, pLA2917, was also mobilized into BYN2 and BYN4 and, as expected, was unable to complement the mutants (Table 2). This was evidence that the *R. sphaeroides* DNA contained in cosmids 473 and 747 is responsible for the change in the phenotype of BYN2 and BYN4.

We also mobilized pMAR1, containing the cloned *cycI* and *adhI* genes found in the overlapping cosmid 473 and 747 DNA, into BYN2 and BYN4. The *cycI* gene encodes isocytochrome c_2 and the *adhI* gene encodes a glutathione-dependent formaldehyde dehydrogenase (10,2). It was unable to complement either of the mutants (data not shown), suggesting that BYN2 and BYN4 are not mutated in the *cycI* or *adhI* genes.

TABLE 2. Complementation analysis of BYN2 and BYN4.

Strain	Cosmid or Plasmid	Growth on	
		SIS + 3-butyn-1-ol	SIS + Tc[a]
2.4.1	—	-	+
BYN2	—	+	+
BYN2	473	-	+
BYN2	747	-	+
BYN2	pLA2917	+	+
BYN4	—	+	+
BYN4	473	-	+
BYN4	747	-	+
BYN4	pLA2917	-	+

[a] Cells were plated onto SIS + Tc to verify the stability of the inoculum.

pLD11 contains the 4.1 kb *Bam*HI-*Eco*RI fragment of pBS4. The attempt to subclone the 4.1 kb *Bam*HI-*Eco*RI fragment of pBS4 into pGEM-3zf(+) produced several possible transformants, each of which were cultured and miniprepped. *Bam*HI-*Eco*RI digests of each plasmid harbored in the transformants showed that pLD11 contains the desired fragment, as the insert from pLD11 co-migrated with the 4.1 kb *Bam*HI-*Eco*RI pBS4 fragment we were attempting to clone (Fig. 5).

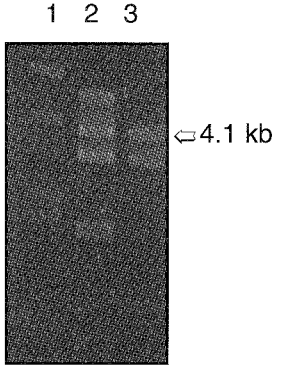

FIG. 5. pLD11 contains the 4.1 kb *Bam*HI-*Eco*RI pBS4 fragment. Lane 1 contains a *Hind*III digest of λDNA used as a size standard. Lanes 2 and 3 contain *Bam*HI-*Eco*RI digests of pBS4 and pLD11, respectively. The desired 4.1 kb fragment is seen in both lanes.

To further verify the clone, *Bam*HI-*Hpa*I and *Bam*HI-*Hpa*I-*Eco*RI digest of pLD11 were prepared (Fig. 6). The *Bam*HI-*Hpa*I digest produced a 2.3 kb fragment corresponding to the flanking *R. sphaeroides* DNA and a 5.0 kb fragment which constitutes 3.2 kb of vector DNA and 1.8 kb of transposon DNA. The *Bam*HI-*Hpa*I-*Eco*RI digest produced 3.2 kb (vector), 2.3 kb (*R. sphaeroides*) and 1.8 kb (Tn*5*) fragments. A diagram of the construction of pLD11 and the location of the IS50 primer used for sequencing is found in Fig. 7.

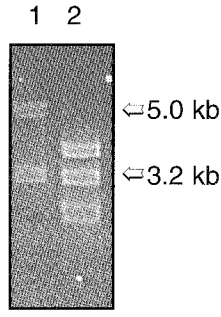

FIG. 6. Verification of restriction enzyme sites within pLD11. Lanes 1 and 2 contain *Bam*HI-*Hpa*I and *Bam*HI-*Hpa*I-*Eco*RI digests of pLD11, respectively.

The mutated gene in BYN4 shows similarity to members of two-component regulatory systems. The sequence obtained from pLD11 is shown in Fig. 8. Protein sequence analysis showed that the product of the ORF that has been interrupted in BYN4 most closely resembles members of two-component regulatory systems from *Bradyrhizobium, Rhizobium,* or *Bacillus* species (Table 3). The closest match was to the Nodulation J Protein from *Bradyrhizobium japonicum*, which showed 41% similarity at the amino acid level (Fig. 9). A second sequencing run using a synthetic primer designed from the original sequence data was unsuccessful.

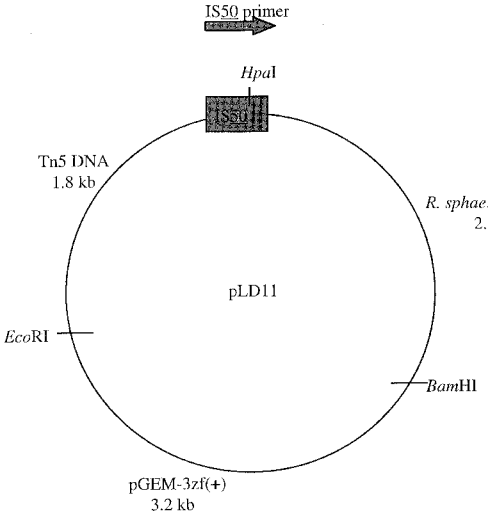

FIG. 7. The construction of pLD11, showing relevant restriction enzyme sites and regions of vector, Tn5 and *R. sphaeroides* DNA. The binding site of the IS50 sequencing primer is also shown.

GNNNNNGNNNNGGTGTGAGAGNCNGATGCTGGNAAAACGGGGAAAGGTTCCGTTCAGGACG

CTACTTGTGTATAAGAGTCAGGCTCTGGGGCATCCGCCCGCCGCCGCTCGGCTATCTCGCCGTC

CTGCCCGCGCTGTTCCTGTCGGGGCTGATGCTGGGCGCGCTCGGCCTCTTCATCTCGTCGGCGA

TCCGGCAGCTCGAGAACTTCGCGGGCGTGATGAACTTCGTGATCTTCCCGATGTTCTTCGCCTC

GACCGCGCTCTATCCGCTGTGGCGGCTGAAGGACTCGAGCCCGCTTCTGGCCGATATCGCGCT

GGCCAATCCCTTCACCCACGCGGTCGAACTGATCCGCTTTGCCCTCTACCGGCAGGTCGAGCCT

possible new primer

CTGGCGCTGGCCGTGGTCGCGGGCGCAGGCCTCGTCTTCTTCGGANCGGCGGTCTTCATGTAC

GATCCCTCGAAGGGCCTGTGGGCCCTGCGCAAGCAATAGGAAGTTCCCATGCTCANATGGTGC

ATTCCGGGCAATCCTCGCGGCGGGCACCGCCATGGGGGCGGAATTTCTCCNATCCCGACTGGN

CCTGCATCCAGCGCAAGCAGCCCCANTTNTTCCCTGGGGCAAGGTCTGGAACNGGCCTCTCCC

GNATCCCCNAACNAAAAACTGGCNCCNAACCGGCAATCCAGGGGTTGGCCANGAACTGNAAA

ACGGCGGNTTCCGTGAAAAAGCCAAAGGGGAATTCCCGTTCCCCCAAACAAACCCAAGAACTG

Genosys primer

TGGCCCCTTTTTCCACCTTGANGGTTTAATNTNCCAACNNTTATNGGGGGCNCCCCTTT

CCNANCCNAAAGCCCCACCCGTNTACCNCCCCCAANAGGCCGTN

FIG. 8. DNA sequence was obtained by using the IS50 primer to sequence out of the transposon and into the flanking *R. sphaeroides* DNA in pLDII. Locations of an unsuccessful synthetic primer made by Genosys Corp. and a possible new primer are shown.

TABLE 3. The mutated gene in BYN4 shows similarity to members of two-component regulatory systems.

Protein	Species	Probability of random match
NodJ	*Bradyrhizobium japonicum*	2.42 x 10_7
NodJ	*Rhizobium leguminosarum* biovar *viciae*	5.31 x 10_7
NatB	*Bacillus subtilis*	5.21 x 10_5
NodJ	*Rhizobium galegae*	6.82 x 10_4
NodJ	*Rhizobium leguminosarum* biovar *trifoli*	9.78 x 10_4

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Db  148  LCAIPTIALTGLVFASLAMVVISLAPTYDYFVFYQSLVLTPMVFLCGAVFPTSQMPDSFQ  207
Qy   28  LAVLPALFLSGLMLGALGLFISSAIRQLENFAGVMNFVIFPMFFASTALYPLWRLKDSSP   87
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FIG. 9. The product of the ORF mutated in BYN4 shows 41% similarity to the Nodulation J protein of *Bradyrhizobium japonicum* at the amino acid level.

DISCUSSION

BYN2 and BYN4 may be mutated in the same region of DNA. The data collected in this study suggest that BYN2 and BYN4 have been mutated in the same region of DNA. Both pBS2 and pBS4 yield a 9.5 kb *Bam*HI fragment which consists of 5.5 kb of Tn*5* DNA and approximately 4 kb of *R. sphaeroides* DNA. In pBS2 the transposon is flanked by 3.3 kb and 1.6 kb of *R. sphaeroides* DNA on either side, while the transposon in pBS4 is flanked by 2.3 kb of *R. sphaeroides* DNA on both sides. This suggests that the transposon has inserted into slightly different positions within the same *Bam*HI fragment of BYN2 and BYN4.

The complementation analyses also suggest that BYN2 and BYN4 are mutated in the same region of DNA. Both mutants are complemented by the wild-type *R. sphaeroides* DNA found within cosmids 473 and 747. In future studies, one side of the *R. sphaeroides* DNA flanking the transposon in pBS2 should be subcloned and sequenced.

Alcohol metabolism in *R. sphaeroides* may be regulated by a two-component regulatory system. Sequence analysis shows that the region of DNA interrupted by the transposon in BYN4 encodes a protein very similar to members of two-component regulatory systems in other organisms. Since the mutation in BYN4 results in a deficiency in alcohol metabolism, as is seen by its resistance to 3-butyn-1-ol, we propose that alcohol metabolism in *R. sphaeroides* is regulated in part by a two-component regulatory system.

Two-component regulatory systems contain a membrane-bound sensor protein and a cytoplasmic DNA binding protein (17). A possible mechanism for such a system's action in *R. sphaeroides* is that the membrane-bound protein binds alcohols in the environment, causing a conformational change in the protein. In turn, this conformational change causes activation of the DNA binding protein which can then turn on transcription of genes responsible for alcohol metabolism. Further studies must be done to elucidate the exact mechanism of this system.

Future direction of this study. One future goal of this work is to obtain additional sequence data. A second sequencing run with a synthetic primer was unsuccessful, possibly due to instability of the region of DNA sequence that the primer was designed from. A different primer, 5' -TTCGGAGCGGCGGTCTTC- 3', comes from a more stable region of DNA and should be tried. Another major objective is to isolate and sequence the exact region of DNA contained within cosmids 473 and 747 that is capable of complementing BYN2 and BYN4. This region should then be probed with DNA from pBS2 and pBS4 to verify hybridization.

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