

Genetic Confirmation of the requirement for PQQ in alcohol metabolism in *Rhodobacter sphaeroides*

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ABSTRACT

Rhodobacter sphaeroides can use many alcohols as carbon and/or energy sources. The breakdown of these alcohols requires enzymes containing cofactors which facilitate electron transfer. Previous research indicates that one type of enzyme used by *R. sphaeroides* for this purpose may be the pyrroloquinoline quinone (PQQ)-containing alcohol dehydrogenase(s). The purpose of this research is to disrupt the PQQ biosynthetic genes in a *R. sphaeroides* strain and to determine if this strain can grow on alcohols. Towards this goal, a cloning strategy has been developed to remove a portion of the PQQ biosynthetic genes and replace this DNA with an antibiotic resistance gene. The plasmids containing both the antibiotic resistance gene and PQQ genes have been purified. Restriction sites for both of these plasmids have been identified and used to isolate desired DNA. Recombinant DNA techniques have been used to construct the desired DNA sequence. The modified version of the PQQ genes will be placed into a strain of *Escherichia coli*, which will be conjugated with *R. sphaeroides*. The mutant *R. sphaeroides* strain will then be tested to confirm exchange of the modified genes. Growth of *R. sphaeroides* on media containing different alcohols as a sole carbon source will be used to determine the role of PQQ genes in the metabolism of various alcohols. In this report, preliminary data is presented and progress towards these goals is discussed.

INTRODUCTION

Alcohols are produced by eukaryotes and bacteria as a result of fermentation. These fermentation processes are necessary to supply energy for growth. Some organisms can produce additional energy by oxidizing these alcohol products (1). For those organisms that can not utilize these alcohols, they become toxic to cells by disrupting cellular membranes (2). In order to prevent death, these cells require enzymes called alcohol dehydrogenases (ADHs).

The metabolic process completed by ADHs requires assistance from cofactors that facilitate electron transfer reactions. NAD(P)⁺-containing ADHs represent one family of these important enzymes. The ADHs of this family contain Zn⁺⁺ and have been well studied (3). The second family consists of pyrroloquinoline quinone (PQQ)-containing ADHs. ADH enzymes belonging to this group have only recently been discovered and have been analyzed in only a few species (4). One of these species is *Rhodobacter sphaeroides*, a Gram-negative bacterium that can be grown aerobically, anaerobically via photosynthesis, or anaerobically with electron acceptors such as dimethyl sulfoxide (5).

This organism is an excellent model system due to its ability to grow under diverse conditions, its unique membrane characteristics, and its ability to use numerous alcohols as carbon and/or energy sources (i.e., n-butanol, methanol, ethanol, and glycerol).

Previous research has led to the isolation of the PQQ biosynthetic genes of *R. sphaeroides*. These genes are believed to be in an operon, or at least relatively close together on the chromosome of *R. sphaeroides* (6). The purpose of this research is to disrupt the PQQ biosynthetic genes of *R. sphaeroides* and confirm their role in the metabolism of various alcohols.

MATERIALS AND METHODS

TABLE 1. Strains and plasmids used in this research

Strain Description	Origin
<i>E. coli</i>	
JM83 Contains pHP45	UW-Madison
DH5 α Used for ligations	UW-La Crosse
S17-A	Used for ligations UW-La Crosse
Plasmids	
pHP45	Contains Ω cassetteUW-Madison
pSOZ11	Contains PQQ genes UW-La Crosse
PJRW13	Cloned plasmid UW-La Crosse
PSUP202	Suicide vector UW-La Crosse

Plasmid purifications were performed using alkaline lysis (9). Preliminary sequence analysis of pSOZ11 and pHP45 was done using a computer database, Genebank. All digestions were performed following manufacturer's guidelines, with 10X KGB buffer substituted for recommended buffers. The fragments resulting from digestions were purified using the "GENECLEAN" protocol (10). Ligation and transformation procedures were taken from Winfrey, Rott, and Wortman (11). Screening for the desired clone was done using antibiotic resistance.

RESULTS

Development of cloning strategy: A cloning strategy was devised (see FIG. 1) based on computer analysis of sequences and previously known information.

Purification of desired plasmid fragments: pSOZ11 and pHP45 were digested using *Bgl* II and *Bam*H I, respectively. A 6.6 kb fragment of pSOZ11 (see FIG. 2), and a 2.0 kb Ω cassette of pHP45 (see FIG. 3) were purified.

Ligation of fragments to create a plasmid, pJRW13: The purified fragments were ligated together creating the new plasmid, pJRW13. The transformation was confirmed using antibiotic resistance, in which the mutants were resistant to both spectinomycin and ampicillin (see FIG. 1). In addition, the plasmid was digested with *Bam*H I and *Hind* III to confirm banding pattern based on known cleavage sites (see FIG. 3 and FIG. 4).

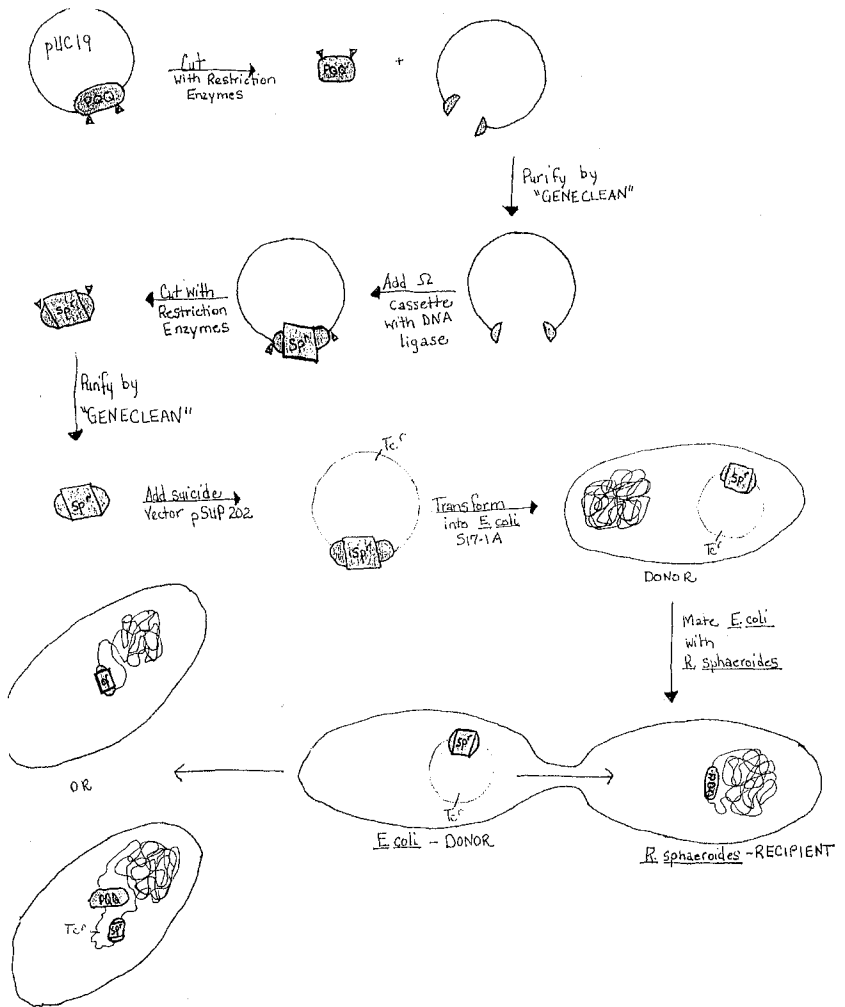


FIG. 1 Diagram of protocol devised to disrupt the PQQ operon. Sp^r is spectinomycin resistance, and Tc^r is tetracycline resistance.

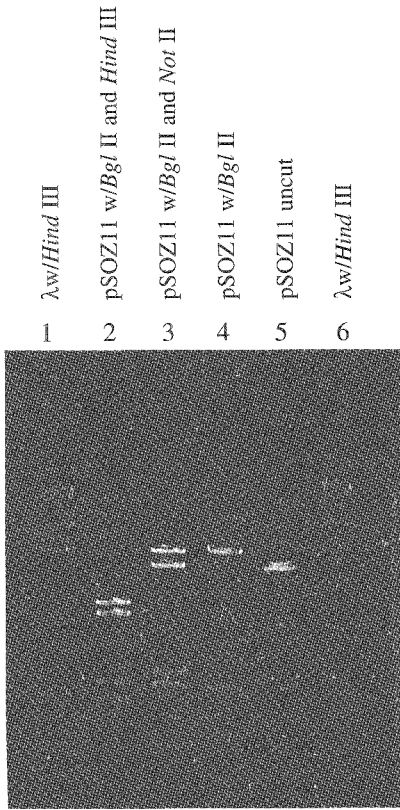


FIG. 2 Gel electrophoresis of pSOZ11, derivative of the plasmid pUC19, containing the PQQ genes. The 6.6 kb *Bgl* II fragment (see lane 4) was purified and used for ligation with pHP45.

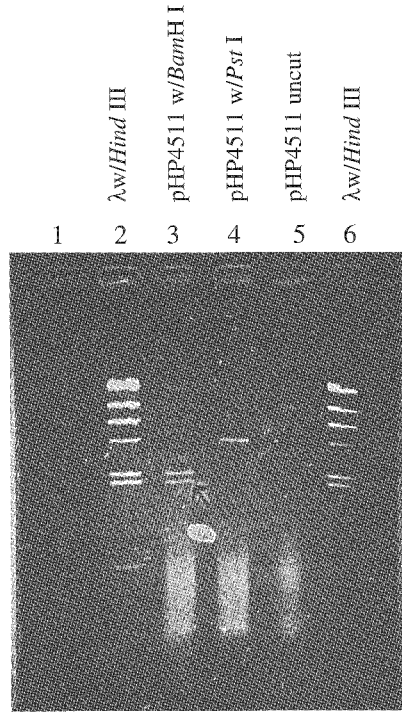


FIG. 3 Gel electrophoresis of a pHP45. The 2.0 kb *Bam*H I fragment (see lane 2) contains an Ω cassette and was used for ligation with pSOZ11.

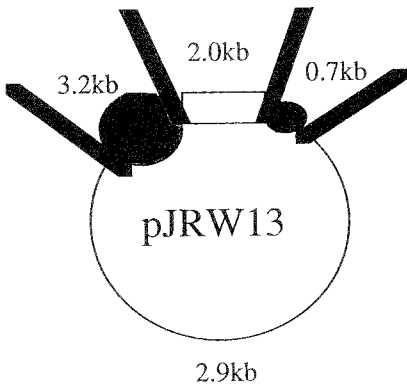


FIG. 4 Diagram of pJRW13 showing *Hind* III and *Bam*H I restriction sites.

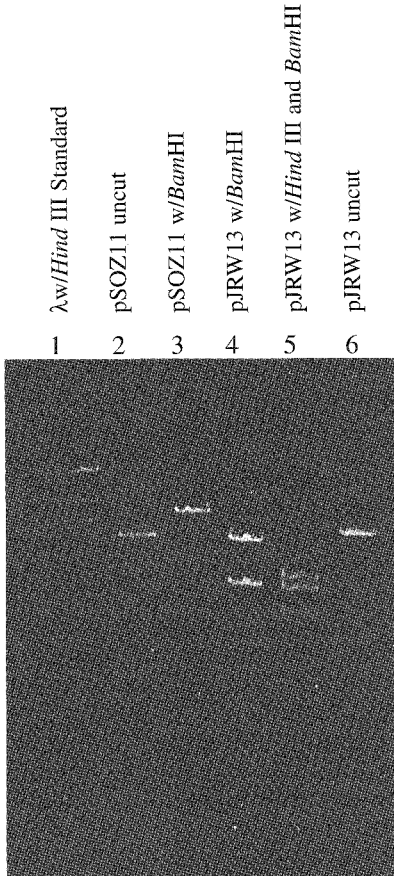


FIG. 5 Gel electrophoresis of pJRW13 with *Hind* III and *Bam*HI. Lane 5 shows the banding pattern of pJRW13 when cleaved with both enzymes (see FIG. 3).

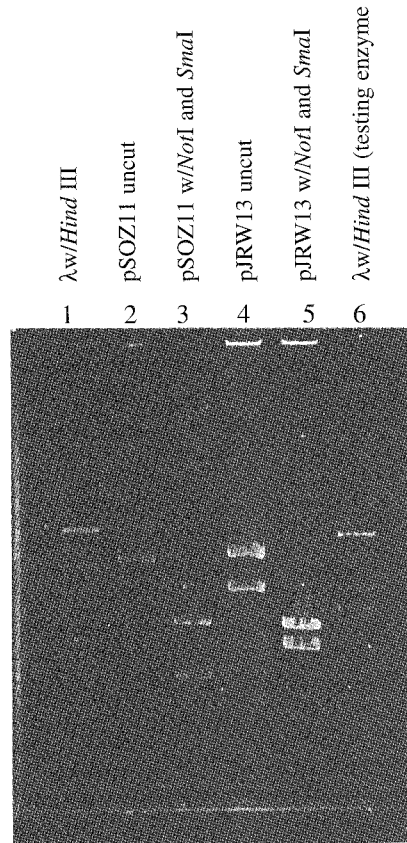


FIG. 6 pJRW13 digested with *Not* I and *Sma* I (see lane 5)

Digestion of pJRW13 and pSUP202 to create suicide vector: pJRW13 was digested using *Not* I and *Sma* I producing a 4.2 kb fragment (see FIG. 5). This fragment is currently being ligated with the suicide vector, pSUP202, for use in further transformations.

DISCUSSION

pHP45 from *E. coli* JM83 was unable to be purified using a large scale prep for reasons still unknown. A small scale prep was used, but resulted in poor yields which led to problems achieving the desired concentrations for the ligations that followed.

The pSOZ11 and pHP45 ligation was initially unsuccessful due to a number of possibilities. As stated above the concentration of the pHP45 fragment was extremely low making it difficult to get a desired ratio between fragments. In addition, when the

“GENECLEAN” procedure is performed, the fragment must be cut out of a gel. In the first two ligations the band was removed after staining it with ethidium bromide and exposing it to UV light. The ethidium and UV light are both mutagens which could have affected the small amount of DNA present in the band. The final ligation used a fragment that was removed without exposure to either mutagen. This ligation was successful and produced the plasmid pJRW13, which contained some of *R. sphaeroides*’ PQQ genes interrupted by the Ω cassette.

Currently, pJRW13 is being digested to remove the portion of PQQ genes containing the Ω cassette. This 4.2 kb fragment will then be placed into the suicide vector, pSUP202, and transformed into *E. coli* S17-A. This strain of *E. coli* is able to conjugate with *R. sphaeroides*, transferring the suicide vector. Once *R. sphaeroides* has taken up the new plasmid either a double cross over will occur and the modified PQQ genes from the suicide vector will replace the wild-type PQQ genes, or the single cross over will occur and the entire suicide vector will be incorporated into *R. sphaeroides*’ genome. Using antibiotic markers on the suicide vector, the type of crossing over event that took place can be determined. Once the correct mutant is obtained, it will be tested on media containing varying alcohols as its carbon source.

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