Site-directed mutagenesis of hydrogenase genes in *Azotobacter chroococcum*

by

Donald Tito

Department of Microbiology
McGill University
Montréal
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### Abbreviations used in the text

#### General:
- **Hup** hydrogen uptake (oxidation)
- **X** any amino acid residue

#### Antibiotics:
- **Amp** ampicillin
- **Cam** chloramphenicol
- **Kan** kanamycin
- **Nal** naladixic acid
- **Spc** spectinomycin
- **Str** streptomycin
- **Tet** tetracycline
- **r/s** (antibiotic) resistance / sensitivity

#### Other reagents:
- **BS** Burk's sucrose (medium)
- **CIP** calf intestinal phosphatase
- **CTAB** cetyltrimethylammonium bromide
- **DEAE** diethylaminoethyl
- **DTT** dithiothreitol
- **LB** Luria-Bertani (medium)
- **NTG** N-methyl-N'‑nitro-N-nitrosoguanidine
- **RM** rich medium

#### Genetic nomenclature:
- **::** novel junction (fusion or insertion)
- **Δ** deletion
- **kb** kilobase(s) or 1000 bp
- **ORF** open reading frame
- **p** plasmid, e.g., pBR322
- **_p** promoter, e.g., lacZ p
Abstract

Accessory hydrogen uptake genes have been identified in a region of the Azotobacter chroococcum genome about 5 kb downstream of the hydrogenase structural genes (hupSL). DNA sequencing has revealed six genes (hupABYCDE) in this region. These genes are probably transcribed in the same direction as hupSL but are probably in a different operon. Mutational analysis had shown that disruption of the hupB, hupY, hupD and hupE genes gives a Hup⁻ phenotype. In the present work additional mutational analysis, using Tn5, a Tn5-derivative containing a promoterless lacZ gene, and a kanamycin resistance gene, confirms the direction of transcription and the separate nature of the hupABYCDE operon, and extends the region known to be necessary for Hup activity to hupA and possibly to 1.6 kb upstream of hupA.
Resumé

Des gènes accessoires à l'oxydation de l'hydrogène ont été identifiés dans une région de génome Azotobacter chroococcum à peu près 5 kb en aval des gènes structuraux de l'hydrogénase (hupSL). La détermination de la séquence de l'ADN a permis de révéler six gènes (hupABYCOE) dans cette région. Ces gènes sont probablement transcrits dans la même direction que hupSL mais sont probablement sur un opéron différent. L'analyse mutationnelle a montré que l'interruption des gènes hupB, hupY, hupD et hupE donne un phenotype Hup-. Une autre analyse mutationnelle basée sur Tn5, un dérivat de Tn5 qui contient un gène lacZ sans promoteur et un gène résistant à la kanamycine a confirmé la direction de transcription et la nature différente de l'opéron hupABYCOE et étend la région qui est reconnue être nécessaire à l'activité de Hup à hupA et possiblement à 1.6 kb en amont de hupA.
1. Introduction

Hydrogenases catalyze the oxidation or evolution of hydrogen, and are widely distributed in nature (Adams et al., 1981). Many N₂-fixing aerobic bacteria, including a minority of rhizobia, have uptake, or H₂-oxidizing, hydrogenases (Evans et al., 1987; Stam et al., 1987). Dixon (1972) suggested that a role for uptake hydrogenases in N₂-fixing legume symbioses could be to recover some of the energy lost through H₂ evolution by nitrogenase. Evans (1987) has reviewed data supporting this idea. The structural genes (hupSL) coding for the uptake hydrogenases in Bradyrhizobium japonicum and Azotobacter chroococcum (Sayavedra-Soto et al., 1988; Ford et al., 1990) have been identified and sequenced, and have been found to be very similar (Ford et al., 1990). A region of the A. chroococcum genome close to hupSL has been found to be essential for the expression of active hydrogenase (Tibelius et al., 1987). Mutagenesis studies and DNA sequencing of this region suggest the presence of an operon separate from that of hupSL which contains at least six genes (hupABYCDE) (Yates and Robson, 1985; Tibelius et al., 1987; Tibelius and Yates, 1989; Tibelius et al., 1993). The proteins putatively coded for by these genes have been compared with homologous proteins found in B. japonicum (Tibelius et al., 1993; Menon et al., 1990 and 1992). However the specific functions of these genes remain unknown. In the present work site-directed mutations were made in this hup-specific DNA using Tn5, a Tn5-derivative containing a promoterless lacZ gene, and a Kan^R gene. The data obtained complement other data (Du et al., 1992; Tibelius et al., 1993) showing that the operon containing hupABYCDE is probably unlinked to hupSL, that the hupABYCDE operon is transcribed in the same direction as hupSL, and that the promoter for this operon probably lies within 1.6 kb upstream of hupA.
2. Literature Review

2.1. Hydrogenases

Hydrogenases (EC class 1.12) are widely distributed in nature and catalyze the reaction described by the equation:

\[ H_2 = 2H^+ + 2e^- \]  \hspace{1cm} (1)

While isolated enzymes can be made to catalyze both the forward, H2 uptake, or H2 oxidation, reaction and the reverse, H2 evolution, reaction in vitro, it appears that the physiological role of any particular enzyme in vivo is limited to either the forward or reverse reaction of eqn. (1) (Adams et al., 1981; O'Brian and Maier, 1988). Many fermentative anaerobic bacteria have H2 evolving hydrogenases which reduce protons in order to oxidize reduced electron carriers active in substrate-level phosphorylation (Adams et al., 1981).

*Escherichia coli*, for example, can ferment fumarate in this way. Other anaerobes use uptake hydrogenases to exploit H2 as an energy source to drive ATP coupled electron transport (Adams et al., 1981). *Desulfovibrio* spp. can oxidize H2 using SO42- as the respiratory electron acceptor. The heterogenous group known as the H2-oxidizing aerobic bacteria are able to grow autotrophically on H2 and CO2. These bacteria may contain either or both of two distinct types of uptake hydrogenases. One type is attached to the membrane, and channels electrons into the respiratory chain; the other type is active in the cytoplasm, where it couples H2 oxidation to NAD reduction (Adams et al., 1981). *Alcaligenes eutrophus* is probably the most thoroughly studied of the H2-oxidizing aerobic bacteria. Certain spp. of cyanobacteria may also contain two uptake hydrogenases, one membrane bound, and a soluble type that reduces NADP (Houchins, 1984). Many nitrogen-fixing aerobes also contain uptake hydrogenases.
2.2. Physiological role of hydrogenases in N$_2$-fixing aerobes

The physiological role of hydrogenases in N$_2$-fixing aerobes appears to be to recycle some or all of the H$_2$ produced by nitrogenase. The reduction of atmospheric nitrogen to ammonia is accompanied by the obligate co-reduction of protons to molecular hydrogen, during the complex catalytic cycle of nitrogenase (Lowe et al., 1984).

The overall equation for this reaction is:

\[ \text{N}_2 + 8 \text{H}^+ + 8 \text{e}^- + 16 \text{ATP} = 2 \text{NH}_3 + 16 \text{ADP} + 16 \text{Pi} + \text{H}_2 \]  

(2)

The stoichiometry of eqn. (2) suggests that 25% of the electron flow is channelled to H$_2$ evolution. Simpson and Burris (1984) reported that even under extreme conditions designed to favour electron flow to N$_2$ (50 atm N$_2$), 27% of the nitrogenase electron flux still went into H$_2$ evolution. Lowe et al. (1984) suggested that the catalytic mechanism of nitrogenase may also allow for proton reduction in the absence of N$_2$-reduction. In a recent review Evans et al. (1987) summarized conditions known to effect the partitioning of electrons between these two substrates in vitro. Dixon (1967, 1968, 1972) found that pea (Pisum sativum) nodules containing Hup$^+$ Rhizobium leguminosarum bacteroids could recycle most, if not all, of the H$_2$ evolved by nitrogenase. However, Schubert and Evans (1976) reported that H$_2$ evolution from most legume nodules typically accounts for one-third to one-half of total nitrogenase electron flux. Dixon (1972) postulated that where nodule bacteroids contain effective hydrogen uptake systems, these may benefit the efficiency of nitrogen fixation in three ways. First, at least some of the energy lost by H$_2$ evolution can be recovered. Eqn. (2) shows that N$_2$ fixation is an energy intensive process, and it is thought that the supply of photosynthate to roots can often limit this reaction. Second, by reducing the partial pressure of H$_2$ around
nitrogenase, product feedback inhibition will be reduced. Third, nitrogenase is quickly and irreversibly inactivated by O₂. By reducing the partial pressure of O₂ around nitrogenase the O₂-dependent respiration of H₂ may offer nitrogenase some protection. Evans (1987) reviewed data from field trials comparing Hup⁺ and Hup⁻ strains of *Rhizobium* and *Bradyrhizobium*, and argued that the weight of evidence supports the conclusion that effective hydrogen recycling is a desirable agronomic trait. In particular an experiment was cited in which soybeans (*Glycine max*) were inoculated with Hup⁺ and Hup⁻ *Bradyrhizobium japonicum* strains that were otherwise isogenic. Plants inoculated with the Hup⁺ strain showed significantly greater nitrogen content and yields. In chemostat studies with *Azotobacter chroococcum*, Yates and Campbell (1989) found that Hup⁺ strains were at a competitive advantage over Hup⁻ strains when organic carbon or phosphate were limiting, but were at a disadvantage when O₂, SO₄²⁻, or iron were limiting. Stam *et al.* (1987), in a theoretical discussion of the energetics of N₂ fixation and H₂ recycling, argued that H₂ recycling is probably not very important, and may be a disadvantage to bacteroids under O₂ limited, carbon sufficient conditions. They allowed that the potential for autotrophic growth on H₂ and CO₂ of free-living *Rhizobium*, between infection cycles, may be of survival benefit for Hup⁺ spp. Stam *et al.* (1987) noted that most *Rhizobium* strains so far tested are Hup⁻, and they suggest that this argues against an ecological advantage for this trait.

### 2.3. Biochemistry of hydrogenases in N₂-fixing aerobes

Hydrogenases have been purified from the aerobic N₂ fixers *B. japonicum* (Harker *et al.*, 1984) and *Azotobacter vinelandii* (Seefeldt and Arp, 1986). A similar enzyme has been purified from the photosynthetic bacterium
Rhodobacter capsulatus (Seefeldt et al., 1987). These are membrane-bound αβ dimers, containing Ni and Fe. The small and large subunits have molecular weights of 31 and 67 kDa, respectively. The larger subunit has been shown to be catalytically active as a monomer, however the individual roles of the subunits are not known (O'Brian and Maier, 1988). Work with membrane preparations from Azotobacter vinelandii has shown the participation of b, c, and d type cytochromes (Wong and Maier, 1984), and ubiquinone (O'Brian and Maier, 1985), in electron transport from H₂ to O₂. In Bradyrhizobium japonicum ubiquinone and type a, b, c, and aa₃ cytochromes have been shown to be active in H₂-dependent electron transport (O'Brian and Maier, 1988).

2.4. Genetics of hydrogenases in N₂-fixing aerobes

2.4.1. Isolation of hup genes from B. japonicum

Cantrell et al. (1983) found cosmids in a B. japonicum gene bank that, when conjugated into B. japonicum Hup⁻ point mutants, could complement these mutations, restoring hydrogenase activity. Lambert et al. (1985) showed that additional B. japonicum DNA was required for Hup activity in free-living cells, and they isolated more cosmids by complementing Tn5 insertion mutants. One of these cosmids, pHU52, carrying approximately 28 kb of Bradyrhizobium DNA, appears to contain all the genetic information necessary for Hup activity in the free-living state. Interestingly, this cosmid was also able to complement Hup⁻ Rhizobium meliloti and R. leguminosarum strains.
2.4.2. Identification of hup DNA in A. chroococcum

Yates and Robson (1985) produced a series of Hup⁺ A. chroococcum mutants by random mutagenesis with NTG. These mutants were classed into four groups based on their Hup phenotype (Yates and Robson 1985; Tibelius and Yates, 1989). Class a mutants had very low Hup activity and had no discernible H₂ evolution activity. Class b mutants had very low Hup activity and evolved some H₂. Class c mutants had low, but significant Hup and H₂ evolution activities. The single class d mutant produced a hydrogenase similar to the solubilized wild type enzyme in O₂ sensitivity, sedimentation behaviour, and pH optimum. Conjugation of pHU1 (Cantrell et al., 1983), a plasmid that contains approximately 22 kb of the B. japonicum DNA carried on pHU52 (Lambert et al., 1985), into Hup⁺ A. chroococcum strains complemented four separate class a mutants. Tibelius et al. (1987) identified A. chroococcum genomic DNA homologous to B. japonicum hup DNA by probing a genomic library with hup specific DNA from pHU1 (Figure 2.1). Plasmids containing this DNA were able to complement some A. chroococcum Hup⁺ mutants (Tibelius et al., 1987). Tibelius and Yates (1989) extended the analysis of the A. chroococcum hup genes by complementing the mutants obtained by Yates and Robson (1985) with various restriction fragments of hup DNA from B. japonicum and A. chroococcum (Figure 2.1). Two regions of hup DNA were identified, covering a total of approximately 15 kb. One region complemented the class b mutants and another complemented the class a and c mutants, suggesting that at least two transcriptional units were present.
Figure 2.1. Physical and genetic map of *A. chroococcum* hup region. The hatched bar above the restriction map shows the sequence identified by *B. japonicum* hup gene probes. The lines directly above the map indicate the fragments complementing NTG mutants of classes b, a and c. The stippled bars indicate the sequences identified by an *A. eutrophus* hoxDE gene probe. The genetic map shows the locations of *A. chroococcum* hup genes so far identified. The solid arrow shows the confirmed direction of transcription. The open arrow shows the presumed direction of transcription. *B*, BamHI, *E*, EcoRI, *G*, BglII. The restriction sites are numbered starting from the BglII site (G1) upstream from hupSL.
2.4.3. Mutagenesis and sequencing of *A. chroococcum* 

*hup* structural genes

Ford *et al.* (1990) found the *A. chroococcum* hydrogenase structural genes (*hup*SL) by probing cloned fragments of *A. chroococcum* DNA with synthetic oligonucleotides, based on the amino-terminal sequence of purified *A. vinelandii* HupS and HupL, and determined the nucleotide sequences of these genes. They compared the predicted amino acid sequences with homologous sequences from the N₂-fixing aerobes *B. japonicum* (Sayavedra-Soto *et al.*, 1988) and *R. capsulatus* (Leclerc *et al.*, 1988) along with those from the chemolithotrophic anaerobes *Desulfovibrio baculatus* and *D. gigas*. These two *Desulfovibrio* spp. also contain NiFe hydrogenases, which are located in the periplasm. In each case the putative protein product of *hupS* contains a signal sequence for membrane attachment of from 25 to 46 residues located at the amino terminus. Menon *et al.* (1990) also determined the nucleotide sequence of homologous genes in *A. vinelandii*. They noted that *hoxK*, which is the homolog of *hupS*, codes for a 45 residue sequence that is absent in the purified protein subunit, and also appears to be a signal sequence. Ford *et al.* (1990) cite other structural aspects of the putative HupS proteins also suggesting membrane association, and give evidence for a Ni binding site on HupL. *A. chroococcum* HupL showed high identities of 64 and 63% with homologous proteins in *B. japonicum* and *R. capsulatus*, respectively; and much lower identities of 38 and 26% with *D. baculatus* and *D. gigas*, respectively. Ford *et al.* (1990) produced *A. chroococcum* MCD1-derived strains by transposon insertions into *hup*SL. These strains had the same Hup⁻ phenotype as the class b mutants made by Yates and Robson (1985).
2.5. Identification, analysis, and mutagenesis of accessory hup genes in A. chroococcum

Du et al. (1992) and Tibelius et al. (1993) identified six putative hup genes by DNA sequencing several kb 3' to the hupL stop site (Figure 2.1). Four of the genes are on fragments that hybridize to the A. eutrophus hoxDE genes (Du et al. 1992; Tibelius et al., 1993). In A. eutrophus these genes seem to be involved in hydrogenase regulation and in processing Ni into the hydrogenase apoenzyme (Eberz and Friedrich, 1991). The putative peptide products of hupD and hupE are very similar to the proposed E. coli hypD and hypE gene products (Du et al. 1992, Lutz et al., 1991). The functions of hypD and hypE are not known (Lutz et al., 1991), but mutations in the E. coli hypABCDE operon have pleiotropic effects on the expression of all three E. coli hydrogenase isoenzymes. Tibelius et al. (1993) found four additional open reading frames (ORFs) in a DNA sequence beginning 3.4 kb upstream of hupD in A. chroococcum. Analysis of the peptides putatively produced by three of these genes, hupA, hupB, and hupC, indicated that they were homologous to the proposed products of E. coli hypA, hypB, and hypC, respectively. The fourth ORF found by Tibelius et al. (1993) has no known homolog in E. coli, and was named hupY. The hupABYCD genes are tightly clustered, and are likely located on a single operon (Du et al., 1992, Tibelius et al., 1993). As noted above, Tibelius and Yates (1989) reported data supporting the idea of two distinct complementation regions of A. chroococcum hup DNA. Du et al. (1992) continued this analysis by attempting to complement mutations in hupS (MCD201, Ford et al., 1990) and hupE (MCD308, Du et al., 1992) with separate fragments of cloned DNA. The 9.2 kb BglII fragment carried in pKHT30 was only able to restore Hup activity to MCD201. The 10.5 kb
BglII - BamHI fragment carried on pKHT33 was only able to restore Hup activity to MCD308. This data showed that at least hupE was transcribed from a promoter different from, or in addition to, that of hupSL.

Du et al. (1992) also obtained mutations in hupD and at 13.9 kb downstream from hupL (Figure 2.1), yielding strains with Hup−, and Hup+ phenotypes, respectively. A double mutant was obtained by transferring a hupD mutation into strain MCD208. MCD208 carries a lacZ gene inserted at the end of hupL that runs off the hupSL promoter. The insertion in hupD carries a transcriptional terminator, and so it should interrupt transcription of hupD and therefore block expression of any downstream genes running off the same promoter such as hupE. The double mutant, MCD311, expresses β-galactosidase activity only slightly greater than that of MCD208, showing that under the conditions tested neither hupD nor hupE are involved in the regulation of transcription of hupSL. The strain MCD312 carries an insertion approximately 2.8 kb downstream from hupE, and is Hup+. This locus of insertion limits the region of the operon containing hupDE which is necessary for Hup expression under the conditions tested by Du et al. (1992). Tibelius et al. (1993) reported that mutations in hupY and hupB also had little effect on the transcription of hupSL, and strains MCD313 and MCD314, carrying these single mutations in hupY and hupB, respectively, were Hup−. Complementation by the 10.5 kb BglII - BamHI fragment carried on pKHT33 was able to restore Hup activity to MCD313, showing that this 10.5 kb fragment contains a promoter responsible for the transcription of at least hupYCD. Accessory, downstream hup genes have also been found in R. capsulatus (Xu and Wall, 1991, Richaud et al., 1991), and A. vinelandii (Menon et al., 1992; Chen and Mortenson, 1992a; Chen and Mortenson, 1992b).
Tibelius et al. (1993) compared the peptides putatively encoded by these genes, and provided insight into the possible protein functions. Figure 2.2 shows the relative clustering of homologous genes from these organisms. The suggested structures for HupA and HupB both have potential metal binding sites, and HupB in particular may contain a Ni-binding motif. No likely function could be suggested for the predicted HupC, a small (84-aa), acidic peptide. The predicted HupY protein has a homolog only in A. vinelandii. The deduced structure of the HupY protein has two likely metal binding domains, a His-rich region near the carboxy terminus, and two Cys-X$_2$-Cys-X$_{18}$-Cys-X$_2$-Cys clusters near the amino terminus similar to Zn-finger motifs found in certain eukaryotic transcriptional activators.
Figure 2.2. Organization of accessory *hup* genes in *A. chroococcum* and of homologous sequences in other organisms. Arrows indicate the direction of transcription. Vertical lines indicate homologous genes.

From Tibelius et al. (1993).
3. Methods

3.1. Bacterial Strains

Bacterial strains used are listed in Table 3.1. *E. coli* strains were routinely grown on Luria-Bertani (LB) medium at 37°C under air. Medium for plating was solidified with 1.2% agar (wt/vol). Broth cultures were usually shaken at 250 rpm. Antibiotics were added as needed to the following final concentrations: ampicillin (Amp), 100 μg/mL; chloramphenicol (Cam), 30 μg/mL; kanamycin (Kan), 25 μg/mL; streptomycin (Str), 10 μg/mL; and tetracycline (Tet), 10 μg/mL. *A. chroococcum* cultures were routinely grown at 30°C on nitrogen-free Burk's sucrose (BS, Newton et al., 1953) medium or on rich medium (RM; Robson et al., 1984). Ammonium acetate was added where needed to a final concentration of 10 mM nitrogen. Antibiotics were added as needed to the following final concentrations: Amp, 50 μg/mL; Cam, 200 μg/mL; Kan, 0.5 μg/mL; naladixic acid (Nal), 20 μg/mL; Str, 10 μg/mL; and Tet, 5 μg/mL. Growth of broth cultures was monitored by spectroscopy of appropriate dilutions of culture samples. *E. coli* culture dilutions were monitored at 600 nm and *A. chroococcum* culture dilutions were monitored at 540 nm. Where increased accuracy of broth culture population estimates were needed, these were obtained by dilution plate counts. For long term storage *E. coli* strains were kept in glycerol-LB (1:1, vol/vol) at -70°C. *A. chroococcum* strains were transferred monthly to fresh agar plates, and these were grown at 30°C until colonies were visible. Afterward they were stored at room temperature.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C600</td>
<td>lacY1  SupE44</td>
<td>Miller (1987)</td>
</tr>
<tr>
<td></td>
<td>Suppressor allows λ573 multiplication</td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>gyrA96 recA1 supE44 Nal r p80lacZ ΔM15 Δ(lacZYAargF)U169</td>
<td>Gibco/BRL</td>
</tr>
<tr>
<td></td>
<td>High transformation efficiency</td>
<td></td>
</tr>
<tr>
<td>ET8000</td>
<td>Nal r</td>
<td>M. G. Yates</td>
</tr>
<tr>
<td>HB101::Tn5</td>
<td>lacY  recA  Str r Tn5 insertion in chromosome</td>
<td>Ditta (1986)</td>
</tr>
<tr>
<td>LE392</td>
<td>lacY1  supE44  supF58</td>
<td>DeBrujin (1987)</td>
</tr>
<tr>
<td></td>
<td>Suppressor allows λ573 multiplication</td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td>recA  Str r  Su o</td>
<td>Bolivar and Backman (1979) Simon et al (1983)</td>
</tr>
<tr>
<td></td>
<td>Contains transfer genes from pRP4 Does not allow λ573 multiplication</td>
<td></td>
</tr>
<tr>
<td>803</td>
<td>Carries conjugal helper plasmid pRK2073</td>
<td>M.G. Yates</td>
</tr>
</tbody>
</table>

1. Characteristics include genetic markers and properties that are relevant to the experiments described in the study.
Table 3.1 continued.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics ¹</th>
<th>Source or reference</th>
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<tr>
<td>A. chroococcum strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCD1</td>
<td>Nal r Str r (wild type)</td>
<td>Robson et al. (1984)</td>
</tr>
<tr>
<td>MCD208</td>
<td>Kan r Nal r Str r Hup - hupL::Tn5 -B20</td>
<td>Ford et al. (1990)</td>
</tr>
<tr>
<td>MCD301</td>
<td>Kan r Nal r Str r Hup - Kan r gene at G9.2</td>
<td>This study</td>
</tr>
<tr>
<td>MCD302</td>
<td>Kan r Nal r Str r Hup - hupA::Tn5 -B20 (G11.0)</td>
<td>This study</td>
</tr>
<tr>
<td>MCD304</td>
<td>Kan r Nal r Str r Hup - hupY::Tn5 -B20 (G14.2)</td>
<td>This study</td>
</tr>
<tr>
<td>MCD305</td>
<td>Kan r Nal r Str r Hup - hupD::Tn5 (G15.0)</td>
<td>This study</td>
</tr>
<tr>
<td>MCD307</td>
<td>Kan r Nal r Str r Hup - hupE::Tn5 -B20 (G15.9)</td>
<td>This study</td>
</tr>
<tr>
<td>MCD309</td>
<td>Kan r Nal r Str r Hup - Tn5 -B20 at G17.6</td>
<td>This study</td>
</tr>
</tbody>
</table>

¹ G9.2, etc. indicates the distance in kb from the BglII site upstream from hupSL (G¹) (Figure 2.1) to the locus of insertion.
3.2. General methods for manipulating plasmids

Enzymes were purchased from Boehringer-Mannheim Biochemica (Laval, Québec), Pharmacia LKB Biotechnology (Baie d'Urfé, Québec), and Promega (Madison, WI).

Plasmids were isolated by the alkaline lysis procedure (Birnboim and Doly, 1979) and were analyzed by restriction endonuclease digestion, and gel electrophoresis in 0.6 to 0.8% agarose (wt/vol) in 1 x TAE buffer (Sambrook et al., 1989), ethidium bromide staining, and visualization by UV-fluorescence.

Restriction enzyme digestions were done by standard procedures (Sambrook et al., 1989) and in accordance with the recommendations of the enzyme manufacturers. Between enzymatic manipulations, DNA solutions were generally in TE buffer (Sambrook et al., 1989).

DNA ligations were done by standard methods (Sambrook et al., 1989). Approximately 0.5 μg of vector DNA with an estimated 3-fold molar excess of insert DNA. The mixed DNA was then precipitated in the presence of sodium acetate and ethanol, and resuspended in 10 μL of water. The mixture was heated to 60°C for 5 min to separate any annealed fragments, and chilled on ice. A 1 μL sample was removed for analysis by gel electrophoresis and 1 μL of 10X ligation buffer was added, adjusting the reaction buffer to: 1 mM ATP, 5 mM DTT, 5 mM MgCl₂, 4 mM spermidine, and 66 mM Tris-hydrochloride (pH 7.6). The ligation reaction was started by the addition of 3 U of T4 DNA Ligase and was allowed to proceed at 16°C, overnight. Another 1 μL aliquot was then removed for analysis by gel electrophoresis, and the ligation reaction was stopped by transferring it to -20°C. In selecting for hybrid plasmid constructs, it was sometimes necessary to prevent recircularization of vector DNA; to do so the 5'-terminal phosphate groups of linearized vector preparations were
removed by treatment with Calf Intestinal Phosphatase (CIP) (Sambrook et al., 1989). Dephosphorylation was done by digesting vector DNA in a reaction volume of 50 μL containing 2 U CIP, 1 mM MgCl₂, 1 mM spermidine, 50 mM Tris-hydrochloride (pH 8.0), and 0.1 mM ZnCl₂. The mixture was incubated at 37°C for 30 min, then 2 more units of CIP were added and the incubation was continued for another 30 min. The reaction was stopped by adding 40 μL water, 10 μL 10X TNE (10 mM EDTA, 1 M NaCl, 100 mM Tris-hydrochloride [pH 8.0]), and 5 μL SDS; and heating to 68°C for 15 min.

Plasmids transformations were done by the CaCl₂ method (Sambrook et al., 1989) using E. coli strain DH5α as host. A 4.5 μL sample of the ligation mixture was added to 100 μL of freshly thawed, competent DH5α held on ice. A 100 μL sample of cold, 100 mM CaCl₂ was then gently mixed in, and the mixture was held on ice for 30 min to allow DNA precipitation on cell surfaces. Transformation was induced by heat shock at 42°C for 90 s. One mL of LB was then added, and the culture was incubated at 37°C for about 1 hr to allow transformed cells to recover from the heat shock. Selection for plasmid uptake was then made by plating culture dilutions on agar plates supplemented with the appropriate antibiotics.

3.3. Cloning hup DNA.

Table 3.2 lists the cloning vectors used and sources of A. chroococcum hup DNA. Table 3.3 lists the various plasmid constructs made during the course of this work.
Table 3.2. Plasmids, bacteriophage, and transposons used.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Precursor</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322</td>
<td>pMB1</td>
<td>Amp r (Tn 3) Tet r (pSC101) 4.36 kb</td>
<td>Bolivar et al. (1977)</td>
</tr>
<tr>
<td>pBR325</td>
<td>pBR322 (pMB1)</td>
<td>Amp r Cam r Tet r 6.00 kb</td>
<td>Bolivar (1978); Prentki et al. (1981)</td>
</tr>
<tr>
<td>pHS1</td>
<td>pEMBL18*</td>
<td>Amp r 4.5 kb E1-E2 hup fragment</td>
<td>Tibelius et al. 1992</td>
</tr>
<tr>
<td>pKHT33</td>
<td>pRK290 (pRK2)</td>
<td>Tet r Mob + IncP 10.5 kb G2-B2 hup fragment</td>
<td>Du et al 1992</td>
</tr>
<tr>
<td>pRK2073</td>
<td>pRK2 (colE1)</td>
<td>Spc r (Tn7) Tra + Conjugal helper plasmid</td>
<td>Ditta (1986)</td>
</tr>
<tr>
<td>pSUP102</td>
<td>pACYC184 (p15A)</td>
<td>Cam r (pR6-5) Tet r (pSC101) Mob + (pRP4) 6.0 kb</td>
<td>Simon et al. (1986)</td>
</tr>
<tr>
<td>pSUP202</td>
<td>pBR325 (pMB1)</td>
<td>Amp r Cam r Tet r Mob + (pRP4) 7.4 kb</td>
<td>Simon et al. (1986)</td>
</tr>
<tr>
<td>pUC4K</td>
<td>pBR322</td>
<td>1.2 kb Kan r gene (Tn903)</td>
<td>Vieira and Messing (1982)</td>
</tr>
<tr>
<td>λ573::Tn5 - B20</td>
<td>λ573</td>
<td>b 221 (att int) c 1857(ts) O 29(am) P 80(am)</td>
<td>Keller et al. (1988)</td>
</tr>
<tr>
<td>Tn5-B20</td>
<td>Tn5</td>
<td>ΔIS50 L::lacZ (ΔlacZp) (pMC1403) Kan r 8.3 kb</td>
<td>Simon et al. (1989)</td>
</tr>
</tbody>
</table>

1. Where known, the sources of particular genetic segments are given parenthetically. 2. B, Bam HI, E, Eco RI, G, Bgl II. The restriction sites are numbered starting from the Bgl II site (G1, Figure 1.1) upstream from hupSL.
3. G9.2, etc. indicates the distance in kb from the BglII site (G1, Figure 2.1), upstream from hupSL, to the locus of insertion.
### Table 3.3. Plasmid constructs.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Vector</th>
<th>Relevant characteristics ¹, ²</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDT1</td>
<td>pBR325</td>
<td>Amp^r Cam^s Tet^r 4.5 kb E₁-E₂ <em>hup</em> fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pDT1.1</td>
<td>pBR325</td>
<td>Amp^r Cam^s Kan^r Tet^r 1.2 kb Kan^r gene insertion at G9.2 on pDT1</td>
<td>This study</td>
</tr>
<tr>
<td>pDT3</td>
<td>pBR322</td>
<td>Amp^r Tet^s 25 kb B¹-B² <em>hup</em> fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pDT3.1</td>
<td>pBR322</td>
<td>Amp^r Kan^r Tet^s, Tn5 insertion at G15.0 on pDT3</td>
<td>This study</td>
</tr>
<tr>
<td>pDT4.1</td>
<td>pSUP202</td>
<td>Amp^r Cam^s Kan^r Tet^r Mob^+ 5.7 kb E₁-E₂ <em>hup::Kan^r</em> fragment from pDT1.1 (insertion at G9.2)</td>
<td>This study</td>
</tr>
<tr>
<td>pDT6</td>
<td>pSUP202</td>
<td>Amp^r Cam^r Tet^s Mob^+ 10.5 kb G²-B² <em>hup</em> fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pDT6.151</td>
<td>pDT6</td>
<td>Amp^r Cam^r Kan^r Tet^s Mob^+, Tn5-B20 insertion at G17.6 on pDT6</td>
<td>This study</td>
</tr>
<tr>
<td>Designation</td>
<td>Vector</td>
<td>Relevant characteristics</td>
<td>Source or reference</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
<td>-------------------------------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>pDT9</td>
<td>pSUP102</td>
<td>Cam(^r)  Tet(^s)  Mob(^+)</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.5 kb  G(^2)-B(^2)  <em>hup</em>  fragment</td>
<td></td>
</tr>
<tr>
<td>pDT9.4</td>
<td>pSUP102</td>
<td>Cam(^r)  Kan(^r)  Tet(^s)  Mob(^+)</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tn5-B20  insertion at G15.9 on pDT9</td>
<td></td>
</tr>
<tr>
<td>pDT9.11</td>
<td>pSUP102</td>
<td>Cam(^r)  Kan(^r)  Tet(^s)  Mob(^+)</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tn5-B20  insertion at G14.2 on pDT9</td>
<td></td>
</tr>
<tr>
<td>pDT9.16</td>
<td>pSUP102</td>
<td>Cam(^r)  Kan(^r)  Tet(^s)  Mob(^+)</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tn5-B20  insertion at G13.2 on pDT9</td>
<td></td>
</tr>
<tr>
<td>pDT9.18</td>
<td>pSUP102</td>
<td>Cam(^r)  Kan(^r)  Tet(^s)  Mob(^+)</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tn5-B20  insertion at G9.3 on pDT9</td>
<td></td>
</tr>
<tr>
<td>pDT9.22</td>
<td>pSUP102</td>
<td>Cam(^r)  Kan(^r)  Tet(^s)  Mob(^+)</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tn5-B20  insertion at G11.0 on pDT9</td>
<td></td>
</tr>
</tbody>
</table>

1. B, *BamHI*, E, *EcoRI*, G, *BglII*. The restriction sites are numbered starting from the *BglII* site (G\(^1\)) upstream from *hupSL* (Figure 2.1).
2. G9.2, etc. indicates the distance in kb from the *BglII* site (G\(^1\), Figure 2.1), upstream from *hupSL*, to the locus of insertion.
In cloning pDT1 (Figure 4.1) the 4.5 kb *hup* fragment was isolated from pHS1 by *Eco*RI digestion and was separated from vector DNA by electrophoresis in 0.8% (wt/vol) agarose in 1x TAE buffer. The gel slab was then turned perpendicular to the current and the *hup* fragment was electrophoretically transferred to a piece of Na45, DEAE cellulose membrane (Schleicher and Schuell, Keene NH) that had been inserted into the gel ahead of the band. DNA was later eluted from the membrane in 1 M NaCl at 65°C following the recommendations of the membrane manufacturer.

In cloning pDT3, pDT6, and pDT9, (Figures 4.4, 4.6, 4.7, respectively) the required *hup* DNA fragments were generated by restriction endonuclease digestion with the appropriate enzymes, and separated from vector DNA by electrophoresis in 0.6% (wt/vol) low melting point (LMP) agarose (Gibco/BRL, Burlington, Ontario) in 1X TAE buffer. Bands containing the required *hup* fragments were cut out of the gel slab and the DNA isolated by glass-bead precipitation using the GeneClean kit (Bio/Can Scientific, Mississauga, Ontario).

### 3.4. Disruption of cloned *hup* DNA.

Tables 3.1 and 3.2 list the mutagenic tools used to disrupt cloned *hup* DNA. Table 3.3 lists the constructs bearing cloned *hup* DNA, and their mutagenized derivatives.

#### 3.4.1. Insertion of a *Kanr* gene

Construct pDT1.1 (Figure 4.2) was made by ligating the 1.2 kb *BamHI* fragment carrying a *Kanr* gene, from pUC4K into the *Bgl* II site of *hup* DNA carried on pDT1.
3.4.2. Insertion of Tn5

Tn5 insertions into the 25 kb BamHI fragment of hup DNA carried on pDT3 were made in the mutagenic host E. coli HB101::Tn5, following the method outlined by Ditta (1986). In this procedure competent host cells bearing Tn5 at an unknown location on the chromosome were transformed with pDT3. The colonies growing on an LB plate supplemented with Amp and Kan were collected together in a few milliliters of LB broth, and plasmids were isolated from this mixture of strains. The plasmid mixture should contain mostly native pDT3 molecules, but may also contain a number of plasmids bearing Tn5 insertions at random locations, excluding loci in essential vector genes. Selection for plasmids bearing Tn5 insertions was done by transforming competent DH5α, and selecting for KanR transformants. Plasmids were extracted from KanR transformants and the loci of Tn5 insertions were mapped by restriction endonuclease digestion and gel electrophoresis.

3.4.3 Insertion of Tn5-B20

Tn5-B20 insertions were made into the 10.5 kb Bgl II - BamHI hup fragment carried on either pSUP202 or pSUP102 (Figures 4.5 and 4.6). The procedure used for mutagenesis was similar to that reported by Keller et al. (1988) and by Simon et al. (1989). The target plasmid was established in E. coli S17-1, a recA, Su0 strain (Table 3.1), by transformation. The plasmid-bearing host was grown to mid-exponential growth phase in LB broth supplemented with 3 mM maltose, to induce the phage λ receptor, and 10 mM MgCl2, to facilitate λ binding. This culture was infected with λ573::Tn5 -B20, at a multiplicity of infection of from 0.1 to 10, and incubated statically for 1 to 2 hr.
Selection for strains bearing the transposon either on a plasmid or on the chromosome was done by plating dilutions of transfected cell culture on LB-Kan agar. Selection for plasmid-borne transposons was done in 2 different ways. 1. Plasmids were recovered from the pooled, transflectant colonies. This heterogenous mixture was used to transform competent *E. coli* DH5α cells, and Kan<sup>r</sup> transformants were selected. 2. Kan<sup>r</sup> transfectants were isolated, and then patched onto LB plates which had been freshly spread with *E. coli* DH5α. Mating was allowed to proceed overnight, and these patches were subcultured on LB plates supplemented with Kan and Nal. Plasmids were isolated from transformant or transconjugant strains with the correct phenotype, and the loci and orientations of transposon insertions were mapped by restriction endonuclease digestion and gel electrophoresis.

### 3.5. Mutagenesis in *A. chroococcum*

Conjugation of plasmids from *E. coli* into *A. chroococcum* was done by two different methods. (1) pDT1.1 was mobilized from *E. coli* DH5α in a triparental mating, as described by Tibelius *et al.* (1987), except that the helper plasmid used to provide transfer functions in the present work was pRK2073 rather than pRK2013. These two plasmids are similar, except that pRK2073 confers Spc resistance whereas pRK2013 confers Kan resistance. Since Kan<sup>r</sup> genes were being transferred into the *Azotobacter* genome it was desirable to have a different antibiotic on the helper plasmid. The *E. coli* donor and helper strains were harvested after growth into stationary phase. The *Azotobacter* recipient strain was harvested during the exponential growth phase. All cells were washed twice in RM and resuspended in RM. Culture samples were mixed together to give an approximate ratio of 1 *E. coli* cell to 10 *Azotobacter*
cells, with donor and helper populations roughly equal. A 100 𝜇l sample of the conjugal mixture was incubated on a nitrocellulose filter disc on RM agar, overnight at 30°C. The heterogenous population of strains was resuspended in RM, and Azotobacter transconjugants were selected by plating dilutions on RM supplemented with Kan, to select for the mutagenic marker, and Str, to select against the E. coli strains. Phenotypic screening against Azotobacter strains retaining vector DNA as a result of a single cross-over event was done by testing for expression of antibiotic resistance genes carried on the vectors. Azotobacter strains showing the correct phenotypes were subcultured at least six times on solid selection medium before further analysis, to allow complete segregation of the multiple copies of the A. chroococcum chromosome (Robson et al., 1984). (2) For all other matings into A. chroococcum the host strain was E. coli S17-1. This strain carries the conjugal transfer functions from RP4 (Simon et al., 1983) on its chromosome, so no helper plasmid is needed. E. coli S17-1 is Str⁻ and Nal⁺, so transconjugant selection was done on RM-Nal. In other respects the two methods used were similar.

3.6. Confirmation of mutant genotypes by Southern hybridization

Genomic A. chroococcum DNA was purified by the method of Robson et al. (1984), except that a step was added which precipitated cell-wall debris, polysaccharides, and protein in 1% (wt/vol) CTAB (Ausubel et al., 1989) and 700 mM NaCl, (final concentrations) after cell lysis. Purified genomic DNA was digested with EcoRI and the resulting fragments were separated on agarose gels and were transferred electrophoretically to nylon filters (GeneScreen, DuPont NEN), which were handled according to the recommendations of the manufacturer. Probes were labelled with Biotin-11-dUTP using a nick
translation kit (Gibco/BRL, Burlington, Ontario); and filters were probed and
developed using a biotin labelling and strepavidin-alkaline phosphatase
detection system (BluGene, Gibco/BRL, Burlington, Ontario) according to the
recommendations of the manufacturer. Hybridizations were carried out under
high stringency at 42°C in 1% (wt/vol) SDS, 10% (wt/vol) dextran sulfate, 45%
formamide, (vol/vol) and 1 M NaCl.
3.7. Enzyme assays

Hydrogenase activity was assayed by the $^3$H$_2$-oxidation method (Llin, 1978), using O$_2$ or methylene blue as terminal electron acceptor. For O$_2$-dependent assays, 1-mL culture samples were transferred to 13-mL serum bottles containing an air atmosphere. The bottles were sealed and pre-incubated for approximately 15 min at 30°C while shaking with a rotational velocity of 250 rpm. The assay reaction was started by adding 1.2 mL of H$_2$ and 0.5 mL of $^3$H$_2$ (specific activities of from 0.6 to 1.2 MBq / mL) to the flask, and the reaction was allowed to proceed for 30 to 60 min at 30°C, with shaking at 250 rpm. The reaction was stopped by transferring 100 μL of sample to 4 mL of scintillation counting fluid. Sample activity was determined by liquid scintillation counting. Methylene blue-dependent assays were done similarly except 0.5 mL culture samples were assayed anaerobically in a final reaction volume of 1.0 mL containing 7.5 mM methylene blue and 100 mM potassium phosphate (pH 8.0).

β-Galactosidase activities were determined as described by Miller (1972). Cultures of A. chroococcum strains bearing promoterless lacZ inserts were harvested during exponential phase growth. The culture was then cooled on ice for about 20 min. A sample of from 0.1 to 0.4 mL of the culture, depending on the culture OD, was transferred to a 1.5 mL micro-centrifuge tube, and the assay volume was adjusted to 0.8 mL with Z buffer (50 mM β-mercaptoethanol, 10 mM KCl, 1 mM MgCl$_2$, 100 mM sodium phosphate [pH 7.0]). Cells were permeabilized with 24 μL chloroform and 40 μL of 0.1% (wt/vol) SDS. Samples were pre-incubated in a 30°C water bath for about 5 min, and the reaction was started by adding 160 μL of o-nitrophenyl-β-D-galactoside (4 mg/mL) in 100 mM sodium phosphate (pH 7.0). The reaction
was then stopped by adding 400 μL of 1 M Na₂CO₃. The tubes were centrifuged for 5 min to remove cells debris from suspension, and the OD₄₂₀ was determined.

3.8. Complementation analysis

The 10.5 kb BglII - BamHI hup fragment had been previously cloned into the broad host range plasmid pRK290 in both orientations, designated pKHT33A and pKHT33B (Du et al., 1992). These plasmids were each mobilized into A. chroococcum MCD302 (G11.0), a Hup⁻ strain, to see if the mutation at G11.0 could be complemented in trans. The resulting strains were assayed for hydrogen oxidation activity using O₂ and methylene blue as terminal electron acceptors by the methods given above.
4. Results and Discussion

4.1. Cloning the 4.5 kb EcoRI hup fragment and its insertional inactivation with a Kanr gene

The 4.5 kb EcoRI hup fragment, isolated from pHS1, was inserted into the unique EcoRI site of pBR325, disabling the the Camr gene. The resulting construct, pDT1 (Figure 4.1), was isolated from Amp', Cam5 transformant colonies. An estimated 97% of the colonies initially isolated on Amp-supplemented media were Camr upon subculture. This indicates that the majority of host cells had received intact pBR325 molecules, probably due to recircularization of vector molecules during the ligation reaction. To clone pDT1.1 (Figure 4.2) The 1.2 kb BamHI fragment containing a Kanr gene was isolated from pUC4K, and inserted into the BglII site (G9.2) of the hup DNA carried on pDT1, eliminating these restriction sites.
Figure 4.1. Cloning of pDT1.

B, BamHI, E, EcoRI, G, BglII. The restriction sites in hup DNA are numbered starting from the BglII site (G1, Figure 2.1) upstream from hupSL.
Figure 4.2. Cloning of pDT1.1.

B, BamHI, E, EcoRI, G, BglII. The restriction sites in hup DNA are numbered starting from the BglII site (G¹, Figure 2.1) upstream from hupSL.
4.2. Cloning of the 25 kb *BamHI-BglII* hup fragment and its mutagenesis with Tn5 and Tn5-B20

In this and later cloning experiments, the linearized vectors molecules were digested with CIP and separated from circular molecules on LMP agarose, to reduce the background of transformation with native vectors. The 25 kb *BamHI* hup fragment from pKHT40 was cloned into pBR322 by inserting it into the *BamHI* site in the Tet\(^r\) gene. pBR322 was used rather than pBR325, despite the advantage of the additional Cam\(^r\) gene, because pBR325 contains a 0.5 kb inverted repeat (Prentki et al., 1981) which might increase the risk of intramolecular recombinations. The resulting construct, pDT3 (Figure 4.3), was isolated from Amp\(^r\), Tet\(^s\) colonies following transfection of *E. coli* DH5\(\alpha\) with the pDT3 ligation mix. Transposon mutagenesis was then done in *E. coli* HB101::Tn5 as suggested by Ditta (1986). Mutagenesis experiments yielded a recombinant plasmid, pDT3.1 (Figure 4.4). The locus of the Tn5 insertion in this construct was mapped, as described in the methods section, at G15.0, in hupD. The frequency of mutagenized plasmids obtained from these experiments was lower than expected using this approach. Some contributing factors limiting the recovery of mutant plasmids may include the following aspects of the experimental method: 1) The population of transposon-bearing plasmids in the pooled mixture purified from a mutagenic host would be expected to be relatively small. DeBruijn and Lupski (1984) noted that transposition of Tn5 from a position in the host chromosome to a resident \(\lambda\) phage gave approximately \(5 \times 10^{-6}\) Kan\(^r\) transductants per phage particle produced. 2) The efficiency of transformation is directly proportional to the size of the construct so the larger transposon-bearing plasmids should enter competent cells at lower efficiency than their smaller precursor molecules.
Figure 4.3. Cloning of pDT3.

B, BamHI, E, EcoRI, G, BglII. The restriction sites in hup DNA are numbered starting from the BglII site (G¹, Figure 2.1) upstream from hupSL.
Figure 4.4. Mutagenesis of pDT3 by Tn5 insertion at G15.

B, BamHI, E, EcoRI, G, BglII. The restriction sites in hup DNA are numbered starting from the BglII site (G1, Figure 2.1) upstream from hupSL.
As transposon mutagenesis in HB101::Tn5 was not yielding satisfactory results, a different approach was utilized.

M.G. Yates (AFRC Institute of Plant Science Research, Nitrogen Fixation Laboratory, University of Sussex, UK) kindly supplied a phage \( \lambda \)-based transposon mutagenesis system developed by Simon et al. (1989). The transposon component of the system, Tn5 -820 (Figure 4.7), is a modified Tn5 carrying a promoterless \( lacZ \) gene in IS50L, which reads into the transposon. This transposon is integrated into a nonessential gene on the chromosome of phage \( \lambda 573 \). Mutations in this phage prevent attachment and integration into the host chromosome, and amber mutations prevent phage reproduction in non-suppressor host strains. The bacterial host for mutagenesis, \( E. coli \) S17-1, is a \( recA \) mutant, that is also \( Str^r \) and \( Su^o \). The initial method chosen for selection of mutagenized plasmids was by transforming \( E. coli \) DH5\( \alpha \) with plasmids purified from the pooled Amp\( ^r \), Kan\( ^r \) colonies obtained following transfection as outlined by DeBruijn (1987). pDT3 was established in \( E. coli \) S17-1, and a number of transfection experiments were done, using \( \lambda ::Tn5 -820 \) at multiplicities of infection from 1 to 10. Plasmids were extracted and examined from approximately 100 Kan\( ^r \) DH5\( \alpha \) colonies isolated by this method. However, none were found with restriction band patterns explainable by a simple transposon insertion. The nature of the events leading to the creation of the recombinant plasmids obtained in these experiments was judged to be outside the scope of the research project and not to warrant further investigation.
4.3. Cloning of pDT6 and its mutagenesis with Tn5-B20

Rather than continue mutagenesis experiments with pDT3, the cloning vector was changed to take advantage of a system developed by Simon et al. (1983) for mobilizing plasmids into Gram-negative bacteria. At the same time it was decided to concentrate on the \textit{hup} region downstream (with respect to \textit{hupSL}) of G9.2, where the first insert had been obtained. The cloning vector we used was pSUP202, a vector derived from pBR325 by the addition of approximately 2.5 kb of RP4 DNA containing the mobilization region (Simon et al. 1983). Although pBR325 had earlier been abandoned as a cloning vector because of its 0.5 kb inverted repeat, this precaution was set aside because of the high mobilization frequency for pSUP202 reported by Simon et al. (1983) with \textit{Rhizobium meliloti}. Low frequency of plasmid mobilization into \textit{A. chroococcum} MCD1 had been a significant problem and it was hoped to obtain better conjugation efficiency with a Mob\textsuperscript{+} vector. The donor strain developed to complement the pSUP plasmids, \textit{E. coli} S17-1, is the same strain used for \textit{\lambda}-based transposon mutagenesis. It contains the trans-acting mobilization functions of RP4 (Simon et al., 1983). pDT6 (Figure 4.5) was constructed by cloning the 10.5 kb \textit{BglII} -\textit{BamHI} \textit{hup} fragment, obtained from pDT3, into the \textit{BamHI} site of pSUP202 (Figure 4.5), disabling its Tet\textsuperscript{r} gene, and eliminating the restriction site at the point of the \textit{hup BglII} insertion. Mutagenesis experiments employing this technique yielded hundreds of putatively mutagenized pDT6-derivatives. Unfortunately mapping of many but not all of these plasmids proved to be difficult, due in part to the lack of precise maps for pSUP202 and Tn5-B20, and more importantly to the unexpected restriction digest band patterns often observed.
Figure 4.5. Cloning and mutagenesis of pDT6. The vertical line under the E^4^-B^2^-hup fragment marks the locus of Tn5-B20 insertion, creating pDT6.151. B, BamHI, E, EcoRI, G, BgIII. The restriction sites in hup DNA are numbered starting from the BgII site (G^1, Figure 2.1) upstream from hupSL.
Preliminary mapping of isolates having restriction band patterns explainable by a simple transposition event generally showed transposon insertions into vector DNA.

One mutagenized plasmid (pDT6.151, Table 3.3, Figure 4.5) was selected for further study. The Tn5 -B20 was found to be integrated in the cloned hup DNA at approximately G17.6, oriented with the lacZ gene reading in the opposite direction of hupSL. In light of the difficulties being encountered, it was again decided to change the cloning vector used for mutagenesis to one that might better facilitate the isolation of a sufficiently large number of mutagenized plasmids to allow the selection of transposon inserts in both orientations, spaced evenly over the entire cloned hup DNA fragment.

4.4. Cloning of pDT9 and its mutagenesis with Tn5-B20

In the first published report of mutagenesis with λ::Tn5 -B20, Keller et al. (1989) noted good results using pSUP102. This plasmid is a Mob⁺, pACYC184-derivative developed by Simon et al. (1986). It was decided to try mutagenesis with this vector, which was kindly provided by the authors.

Construct pDT9 (Figure 4.6) was made by cloning the 10.5 kb BamHI - BgII hup fragment, isolated from pDT3, into the Bam HI site of pSUP102, disrupting the Tet gene. This construct was then established in E. coli S17-1 for Tn5 -B20 mutagenesis. Conjugal selection for plasmid borne transposons was done by mating transfected E. coli S17-1 (pDT9) isolates with E. coli DH5α; and Nal⁻ Cam⁺ Kan⁺ colonies were easily obtained. Although many of the plasmids isolated did not have explainable restriction band patterns, the frequency of transposition into vector DNA seemed to be much less than that observed with pSUP202.
Figure 4.6. Cloning of pDT9.
B, BamHI, E, EcoRI, G, BglII. The restriction sites in hup DNA are numbered starting from the BglII site (G¹, Figure 2.1) upstream from hupSL.
Five mutagenized plasmids (Table 3.3, Figure 4.7) were selected for further study following the determination of the location and orientation of the transposon. In one of these plasmids (pDT9.18, G9.3) lacZ reads in the same direction as hupSL, in the other four plasmids the lacZ gene reads in the opposite direction. While more inserts more evenly representing both orientations were still needed, transposon mutagenesis was being superseded by more convenient methods. Other work in this laboratory was rapidly providing primary sequence data on this DNA; so it was becoming possible to use more restriction sites for insertional mutagenesis by simple cloning methods.
Figure 4.7. Mutagenesis of pDT9. The bars shown inside the representation of pDT9 mark the loci of Tn5-B20 insertions. B, BamHI, E, EcoRI, G, BglII. The restriction sites in hup DNA are numbered starting from the BglII site (G1, Figure 2.1) upstream from hupSL.
4.5 Site-directed mutagenesis of the \textit{A. chroococcum} MCD1 genome

The Azotobacter strains made in this work are listed in Table 3.1.

An attempt was made to transfer the mutation at G9.2, carried on pDT1.1, into the \textit{A. chroococcum} genome. This was done by a triparental mating involving the donor \textit{E. coli} DH5\textalpha{} (pDT1.1), helper \textit{E. coli} 803 (pRK2073), and recipient \textit{A. chroococcum} MCD1. However no recipients of the correct phenotype were isolated.

An attempt to transfer this same mutation into the \textit{A. chroococcum} genome, this time carried on pOT4.1 in \textit{E. coli} S17-1, was successful. The resulting strain was designated MCD301. Conjugation with pDT3.1 (G15.0) gave Azotobacter strain MCD305 by double reciprocal recombination. The mutation carried on pDT6.151 (G17.6) was successfully integrated into the \textit{A. chroococcum} genome, yielding isolate MCD309. However this strain was later lost. The mutation at G13.2 carried on pDT9.16 appeared to be successfully integrated into the \textit{A. chroococcum} chromosome, as Nal\textsuperscript{r}, Kan\textsuperscript{r}, Cam\textsuperscript{s} transconjugant colonies were obtained following matings, and cells showed typical \textit{Azotobacter} morphology. However colonies of this strain were atypical. The strain was unstable and died out after a number of subculturings.

Construct pDT9.18 (G9.3) was integrated into the \textit{A. chroococcum} genome by single rather than double recombination, during many separate conjugation experiments, but the integration of this mutation into \textit{hup} DNA without vector co-integration was not accomplished. It was tried repeatedly to recombine this mutation into the genome because it represents the only construct containing a \textit{lacZ} gene reading in the same direction as \textit{hupSL}. The failure to obtain integration of the mutation alone was likely due to the very
close proximity (approximately 0.1 kb) of the locus of transposon insertion to the limit of the cloned hup fragment.

Constructs pDT9.22 (G11.0), pDT9.11 (G14.2), and pDT9.4 (G15.9) yielded recombinant Azotobacter strains MCD302, MCD304, and MCD307, respectively, by double reciprocal recombination (Figure 4.8).
Figure 4.8. Physical and genetic map of the *A. chroococcum* hup region showing the loci of mutations made during this study. The loci of various mutations are indicated along with the relevant MCD1-derivative strain numbers. Strains MCD301, MCD302, MCD304, MCD305, MCD307, and MCD309 were made during the present work.
4.6. Hydrogen oxidation activities of mutant *A. chroococcum* strains

*A. chroococcum* strains MCD301, MCD302, MCD304, MCD305, and MCD307 were assayed with both O$_2$ and methylene blue as terminal electron acceptor. No Hup activity was observed in any of these strains. These results showed for the first time that a region stretching from about 3.4 to 10.1 kb downstream of *hupSL* was required for hydrogenase activity in *A. chroococcum*. Methylene blue accepts electrons directly from hydrogenase, so the fact that all of these mutants were Hup$^-$ when assayed with methylene blue indicates that the affected loci may be responsible for functions necessary for the formation or activity of the hydrogenase holoenzyme itself. These functions could include enzyme maturation, membrane attachment, catalysis or regulation. Had a mutation given rise to a strain that was Hup$^-$ when assayed with O$_2$, but Hup$^+$ when assayed with methylene blue, that would have suggested that the locus was involved in electron transport. These observations complement other data obtained in this laboratory (Du et al., 1992; Tibelius et al., 1993) showing that disruption of *hupB*, *hupY*, *hupD*, and *hupE* gave Hup$^-$ phenotypes. Each insertion is probably polar, terminating transcription of the operon it is in.

Du *et al.* (1992) reported that MCD312, carrying an insert approximately 3 kb downstream from *hupE*, did not eliminate Hup activity. This indicated that the *hupABYCD*E operon, or that portion of it necessary for Hup activity under the conditions tested, ends before this point. So it would have been interesting to see if strain MCD309, which carries an insert about 2 kb upstream of the insert in MCD312, was also Hup$^+$. Unfortunately MCD309 was the strain that was lost.
4.7. β-galactosidase activities of mutant A. chroococcum strains carrying a promoterless lacZ gene

Table 4.1 shows the β-galactosidase activities of A. chroococcum MCD1 and strains mutated with Tn5-B20. Strain MCD208 carries the transposon towards the end of hupL, with the lacZ gene reading in the same direction as hupSL, and was included as a positive control. The fact that none of the downstream lacZ (ΔlacZ p) gene insertions, which are oriented counter to hupSL, were transcribed, supports sequence data (Tibelius et al., 1993) indicating that the hup genes in this region are transcribed in the same direction as hupSL.
Table 4.1. β-galactosidase activities of *A. chroococcum* MCD1 and derived strains carrying a promoterless *lacZ* gene in accessory *hup* DNA.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Hup phenotype</th>
<th>β-galactosidase activity $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCD1</td>
<td>Wild type</td>
<td>Hup$^+$</td>
<td>0.0</td>
</tr>
<tr>
<td>MCD208</td>
<td>$hupL::$Tn5-B20 (G5.7)</td>
<td>Hup$^-$</td>
<td>48.3</td>
</tr>
<tr>
<td>MCD302</td>
<td>$hupA::$Tn5-B20 (G11.0)</td>
<td>Hup$^-$</td>
<td>2.4</td>
</tr>
<tr>
<td>MCD304</td>
<td>$hupY::$Tn5-B20 (G14.2)</td>
<td>Hup$^-$</td>
<td>0.9</td>
</tr>
<tr>
<td>MCD307</td>
<td>$hupE::$Tn5-B20 (G15.9)</td>
<td>Hup$^-$</td>
<td>0.0</td>
</tr>
</tbody>
</table>

1. Miller units. Activities reported are the means of duplicate assays.
4.8. Trans-Complementation of the mutation at G11.0 with pKHT33

Other studies (Tibelius and Yates, 1989; Du et al., 1992) indicated the presence of at least two complementation units within the approximately 19.6 kb BglII - BamHI fragment (Figure 2.1) that contains both hupSL, and the hup loci investigated in this work. In order to confirm this finding, and better locate the promoters responsible for the transcription of these downstream hup genes, additional complementation studies were done. MCD302 was chosen for complementation since it is Hup - and carries the mutation in a putative downstream hup gene that is the closest to hupSL. This strain was complemented separately with pKHT33A and pKHT33B by conjugation from E. coli S17-1 (Figure 4.8). The complementing plasmids contain the same 10.5 kb hup fragment used to make pDT9, here cloned into pRK290 in both possible orientations (designated A and B). The broad host range plasmid pRK290 can be stably maintained in the cytoplasm of A. chroococcum with selection for Tet'.

$^{3}\text{H}_2$-oxidation assays with $\text{O}_2$ or methylene blue as the electron acceptor showed that establishment of either orientation of the plasmid restored Hup activity to wild type levels (Table 4.2). The presence of complementing DNA was confirmed by plasmid extraction and agarose electrophoresis. This experiment confirms that the 10.5 kb BamHI - BglII hup fragment carries its own promoter, that this promoter is active in A. chroococcum while carried on a plasmid, and that the genes transcribed from this promoter complement the mutation at G11.0 in trans.
Table 4.2. Hup activities of *A. chroococcum* MCD302 and transconjugants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hup activity ¹</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MB ²</td>
<td>O₂</td>
<td></td>
</tr>
<tr>
<td>MCD1</td>
<td>107</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>MCD302</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MCD302 (pKHT33A)</td>
<td>156</td>
<td>227</td>
<td></td>
</tr>
<tr>
<td>MCD302 (pKHT33B)</td>
<td>174</td>
<td>171</td>
<td></td>
</tr>
</tbody>
</table>

1. Specific activities given in nmol of H₂ oxidized / mg of protein per min.

2. Activities varied with the culture age; those reported were the highest observed and are the means of duplicate assays.

4.9. Confirmation of site-directed mutagenesis by Southern hybridization

Genomic DNA was isolated from *A. chroococcum* MCD1 and the Hup- mutant strains described in this work, and probed with appropriate biotin-11-dUTP labelled DNA fragments. *EcoRI* digest fragments of genomic DNA were separated by agarose gel electrophoresis and transferred electrophoretically to nylon membranes, and these were handled as described in the methods section. Figure 4.9 shows the results obtained on developed membranes. Table 4.3 summarizes the expected results. In probing MCD301 genomic DNA we expected to see the loss of the 4.5 Kb band, and the appearance of a mutant 5.7 kb band containing the Kan'' gene. MCD305 genomic DNA was expected to show the loss of the 1.9 kb band and its replacement by a 7.7 kb band, containing Tn5. Tn5-B20 (Figure 4.7) contains an internal *EcoRI* site flanked by approximately 2.9 and 5.4 kb to the left and right, respectively. So *EcoRI* digests of mutagenized genomic DNA should show the loss of one wild type fragment and the appearance of two new fragments each containing a part of the lost fragment plus approximately 2.9 or 5.4 kb of additional DNA. The wild type *EcoRI* bands expected to have been lost in MCD302, MCD304, and MCD307 are 4.5 kb, 2.4 kb, and 1.9 kb, respectively. The expected mutated bands are: MCD302 (4.1 and 8.7 kb), MCD304 (3.4 and 7.3 kb), and MCD307 (3.5 and 6.7 kb). In Figure 4.9, pDT9.4 (Table 3.3, Figure 4.7) DNA was used at the probe. pDT9.4 contains all of the hup DNA mutagenized in this study. The Tn5 DNA in this probe acts as a control against *in vivo* transposition in *Azotobacter* cells. This blot confirmed the expected mutant bands for MCD302 and MCD304. Unfortunately, the wild type bands in the MCD1 digest were not visible on this blot. The remaining mutations have not been confirmed or
negated by Southern hybridization, despite repeated efforts. This was due to difficulties in obtaining pure preparations of genomic DNA, and in some cases to the loss of viability of isolates during subculturing.
Table 4.3. Expected *hup*-specific *EcoRI* digest bands in *Hup*⁻ *A. chroococcum* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lost wild type band</th>
<th>New mutant band(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCD301</td>
<td>4.5 kb</td>
<td>5.7 kb</td>
</tr>
<tr>
<td>MCD302</td>
<td>4.5 kb</td>
<td>4.1 and 8.7 kb</td>
</tr>
<tr>
<td>MCD304</td>
<td>2.4 kb</td>
<td>3.4 and 7.3 kb</td>
</tr>
<tr>
<td>MCD305</td>
<td>1.9 kb</td>
<td>7.7 kb</td>
</tr>
<tr>
<td>MCD307</td>
<td>1.9 kb</td>
<td>3.5 and 6.7 kb</td>
</tr>
<tr>
<td>MCD309</td>
<td>11.5</td>
<td>3.9 and 15.9 kb</td>
</tr>
</tbody>
</table>
Figure 4.9. Southern hybridization of EcoRI digests of genomic DNA from *A. chroococcum* MCD1 and from Hup− strains. Lanes: 1, MCD301; 2, pDT9.4 (bands at 7.3, 6.7, 5.1, 3.5, and 2.4 kb; 3, MCD302; 4, MCD304; 5, MCD307; 6, MCD309; 7, MCD305; 8, MCD1; 9, biotinylated λ HindIII DNA.
5 Cited references


