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ANALYSIS OF *NIF* AND REGULATORY GENES IN *ACETOBACTER DIAZOTROPHICUS*

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Summary—The focus of our collaborative research program is the identification and characterization of *nif* and related regulatory genes of *Acetobacter diazotrophicus* in order to understand what factors influence nitrogen fixation in this unique diazotroph. To date, the following genes have been isolated from genomic libraries and are being analyzed: *nifHDK, nifA, nifB, nifV, nifE* and *ntrBC*. In addition, Nif$^-$ mutants have been constructed by insertional mutagenesis. These mutants are currently being used in inoculation experiments of sterile sugarcane plants to determine whether nitrogen fixed by *A. diazotrophicus* is significant for plant nutrition. © 1997 Elsevier Science Ltd

INTRODUCTION

Acetobacter diazotrophicus is the major diazotroph isolated from the leaves, stems and roots of sugarcane collected in various sites of Brazil (Cavalcante and Dobereiner, 1988), Australia (Li and MacRae, 1991), Mexico (Fuentes-Ramirez *et al.*, 1993), and Cuba (Dong *et al.*, 1994). This bacterium was only isolated from sugarcane and not from roots of other weeds and grasses growing in the same field nor in non-rhizosphere soils suggesting its specific interaction with sugarcane (Boddey *et al.*, 1991; Li and MacRae, 1991). *A. diazotrophicus* is considered a true endophyte based on its initial isolation from surface sterilized tissues and more recently on ultramicroscopic examinations of infected tissues (Cavalcante and Dobereiner, 1988; Dong *et al.*, 1994; James *et al.*, 1994). Other unique characteristics of this Gram-negative bacterium include the ability to grow and fix nitrogen at low pH, production of acetic acid from sucrose or glucose, and growth at high sucrose concentrations (Stephan *et al.*, 1991). It is also the only known diazotrophic species of *Acetobacter*.

There are a number of other diazotrophic bacteria associated with sugarcane but *A. diazotrophicus* may be of particular interest for two reasons: (1) its ability to fix nitrogen is not inhibited by the presence of nitrates (Cavalcante and Dobereiner, 1988), and (2) it is apparently able to transfer 50% of its fixed nitrogen to co-cultured yeast in an in vitro system modeling a plant–bacteria interaction (Cohjo *et al.*, 1993). This observation suggests its potential for contributing significantly to sugarcane N nutrition. We have initiated a program to identify and characterize *nif* and related regulatory genes in order to understand what factors influence nitrogen fixation in the unique habitat occupied by this organism. In addition, we plan to determine whether nitrogen fixed by *A. diazotrophicus* is significant to sugarcane nutrition. This paper presents current progress in our efforts towards these goals.

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MATERIALS AND METHODS

Isolation and sequencing of nif *and other regulatory genes*

Plasmid and phage genomic libraries of *A. diazotrophicus* were constructed in *E. coli* using the wide host range cosmid pLAFR3 and the bacteriophage lambda cloning vector EMBL3. These libraries were screened by ability to complement certain *nif* or *ntr* mutant strains of *Azotobacter vinelandii* (Toukdarian and Kennedy, 1986; Jacobson *et al.*, 1989) or by hybridization to cloned *nif* genes of other diazotrophs. Complementing or hybridizing clones were further analyzed by restriction analysis and fragments were subcloned in pSVB30 and other vectors for sequencing and genetic manipulations. Isolated genes were sequenced by the dideoxy termination method using the Sequenase 7-Deaza-

dGTP DNA Sequencing kit (United States Biochemical, Cleveland, Ohio).

Construction of nif$^-$*-mutants*

Mutations disrupting the isolated genes were constructed by insertion of cassettes carrying kanamycin resistance (*aph*) and the *gus* reporter genes at strategic restriction sites, sometimes resulting in deletion as well as insertion mutagenesis. Mutated genes were introduced back to *A. diazotrophicus* chromosome by conjugation of suicide vectors carrying the mutated gene regions. The resulting transconjugants were grown in a selective medium containing kanamycin, and their Nif phenotype was tested by measuring acetylene reduction and ability to grow on N-free LGI medium.

RESULTS AND DISCUSSION

nifHDK

A 4.3 kb *Hind*III fragment from an EMBL3 library clone hybridized to *nifHDK* DNA from *Azospirillum brasilense*. This fragment corresponded to the 4.3 kb hybridizing region of *Hind*III digested *A. diazotrophicus* chromosomal DNA. This fragment was subcloned from the EMBL3 recombinant and its DNA sequence determined as described in Materials and Methods. This fragment contains a complete *nifD* gene flanked by nearly complete *nifH* and *nifK* genes; approximately 15 base pairs are missing from the 5′ end of *nifH* and 75 base pairs from the 3′ end of *nifK*. Thus *nifHDK* are contiguous and probably cotranscribed as in most other diazotrophs. Data base searches showed that the *A. diazotrophicus nifH* gene product is highly homologous to NifH of other diazotrophs both at the DNA and protein levels. The features common to all other sequenced NifH proteins are also found in *A. diazotrophicus*. For example, the four conserved Cys residues found in diazotrophic Eubacteria are present as well as the conserved regions surrounding these Cys residues. In addition, the 11 amino acids located near the N-terminus which are conserved among known NifH protein sequences are also present in *A. diazotrophicus* (Fig. 1). This region containing the motif Gly-X-Gly-X-X-Gly-Lys-Ser is believed to be an ATP-binding site (Robson, 1984). Data base searches and multiple sequence analysis revealed that based on the *nifH* sequence, *A. diazotrophicus* is closely related to *Rhizobium, Bradyrhizobium, Rhodobacter, Azospirillum* and *Rhodospirillum* (Fig. 2). These bacteria including *A. diazotrophicus* have been grouped in the α-subgroup of Proteobacteria based on 16s rRNA analysis (Young, 1992).

An insertion of the *aph*/*gus* cassette at the *Eco*RI site within the *nifD* gene of *A. diazotrophicus* resulted in the complete removal of the nitrogen-fixing ability of this diazotroph. The isolated mutants were unable to grow in N-free media but grew at the same rate as wild-type in media supplemented with NH_4^+. In addition, mutants were unable to reduce acetylene to ethylene in semi-solid media. These mutants are currently being used in inoculation experiments to establish whether nitrogen fixation by *A. diazotrophicus* contributes significantly to N nutrition in sugarcane.

Acetobacter di	LRQIAFY	GKGGIGKS TTSQNTL
Rhizobium meli	LRQIAFY	GKGGIGKS TTSQNTL
Bradyrhizobium	LRQ IAFY	GKGGIGKS TTSQNTL
Rhizobium legu	LRQ IAFY	GKGGIGKS TTSQNTL
Azospirillum b	LRQ IAFY	GKGGIGKS TTSQNTL
Rhodobacter ca	LRQ IAFY	GKGGIGKS TTSQNTL
Rhodospirillum	LRQ IAFY	GKGGIGKS TTSQNTL
Azotobacter ch	MRQCAIY	GKGGIGKS TTTQNLV
Anabaena sp.	IR Q IAFY	GKGGIG KS TTSQNTL

Fig. 1. Alignment of NifH protein sequences showing the conserved motif near the N-terminus (boxed).

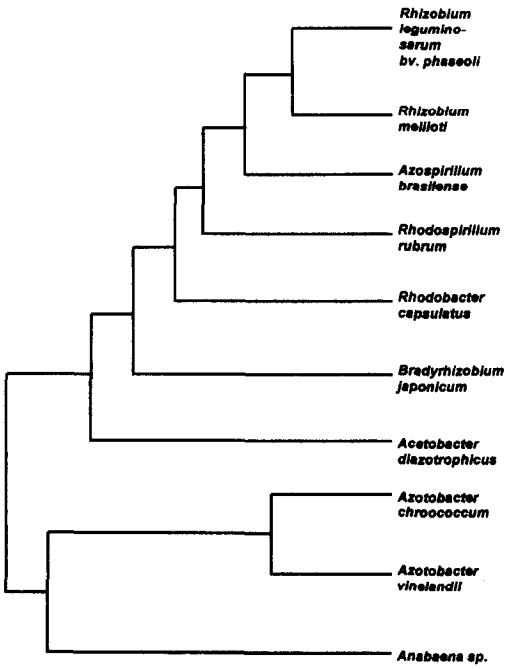

Fig. 2. Dendrogram generated by PILEUP using NifH protein sequences showing the relationship of *A. diazotrophicus* NifH with those of other diazotrophs.

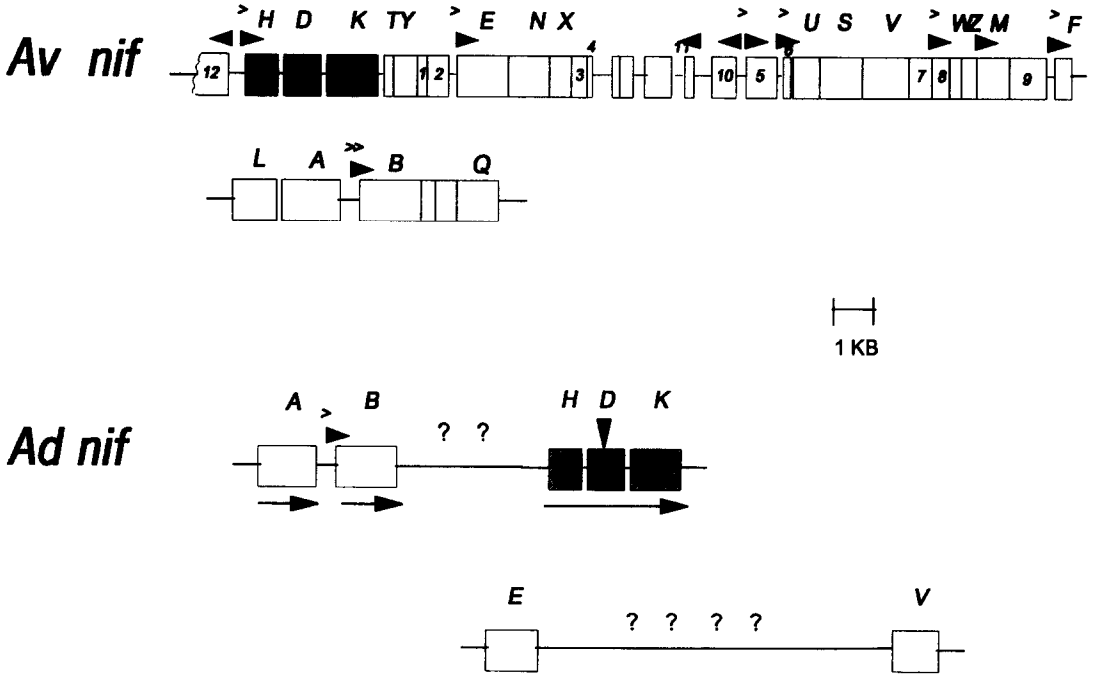

Fig. 3. Arrangement of *nif* genes in *A. vinelandii* (*Av nif*) and those now characterized in *A. diazotrophicus* (*Ad nif*). ▶ indicates σ^{54}-dependent promoters; > indicates NifA binding sites; arrows under *Ad nif* genes indicate their direction of transcription deduced from the DNA sequence. ▼ shows the site of the *gus/aph* insertion cassette.

nifA *and* nifB

A pLAFR3 cosmid clone carrying *nifA* and *nifB* genes of *A. diazotrophicus* was isolated by its ability to complement an *A. vinelandii nifA* mutant. The *nifA* gene was localized on the cloned 22 kb *A. diazotrophicus* fragment by testing the complementing ability of subcloned fragments. Sequencing demonstrated that an open reading frame corresponding to *nifB* is located 165 bp downstream of the end of the *nifA* gene. Interestingly, sequences identical to those obtained for the *nifK* gene were located about 4 kb downstream of *nifA* on the cosmid. The relationship between the *nifHDK* and *nifAB* genes is shown in Fig. 3. The only other diazotroph in which there is a similar distance between *nifHDK* and *nifAB* is *Rhodobacter capsulatus*, but in this organism the *nifHDK* operon lies upstream of *nifAB* rather than downstream as in *A. diazotrophicus* (Ahombo *et al.*, 1986). Sequences identical to the σ^{54} binding site (−24, −12) and NifA recognition motif TGT-N_{10}-ACA were located at positions upstream of *nifB* appropriate for function. Thus, as expected for a diazotrophic member of the alpha group of Proteobacteria, *nif* gene expression in *A. diazotrophicus* is likely to require σ^{54} and NifA. The promoter regions of *nifA* and *nifHDK* have not yet been characterized. The NifA protein is very homologous to those of the other alpha group diazotrophs, including a conserved region between the central and C terminal domains that correlates with the sensitivity of these proteins to oxygen (for review, see Merrick, 1992).

nifV *and* nifE

Several *nif* mutants of *A. vinelandii* were used as conjugal recipients in cosmid transfer experiments, including, in addition to *nifA*, those in *nifH*, *nifE*, *nifN*, *nifU*, *nifS*, *nifV* and *nifM* (Jacobson *et al.*, 1989). Of these latter 7, only the *nifE* and *nifV* mutants were complemented by *A. diazotrophicus* library cosmids. Of great interest is that the cosmids complementing the *nifE* mutant also complemented *nifV* and vice versa; as expected, identical restriction maps were obtained for these cosmids. The location of the genes on the insert DNA was determined by hybridization to plasmids carrying DNA from the *nifV* and *nifE* region of *A. vinelandii*. The results suggest that *A. diazotrophicus nifV* and *nifE* genes are separated by about 8 kb of DNA, a distance similar to that separating *nifV* and *nifE* in *A. vinelandii* (see Fig. 3). The cosmid carrying the *nifV* and *nifE* regions does not appear to overlap those carrying *nifHDK* or *nifAB* described above. Whether the former also carries the other *nif* genes expected to be found between *nifE* and *nifV* as in *A. vinelandii* and *Klebsiella pneumoniae* will be determined by DNA sequencing.

ntrBC

The *ntrBC* genes were isolated by complementation of *ntrC* mutant strains of *A. vinelandii* and

E. coli using the pLAFR3 library. Sequence analysis also revealed a high degree of similarity of these genes to *ntrBC* genes of other bacteria. The *ntrBC* genes of *A. diazotrophicus* are organized in a *nifR3–ntrBC* gene cluster similar to those found in *A. brasilense* and *Rhodobacter capsulatus*. *NifR3* is predicted to encode a protein which is highly similar to that encoded by an open reading frame upstream of the gene encoding *fis*, an *E.coli* DNA-binding protein (Foster-Hartnett *et al.*, 1993). Mutations in these genes are being constructed to study their roles in the regulation of *A. diazotrophicus* nitrogen fixation.

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