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# Cloning of ferredoxin I gene from *Azotobacter vinelandii* using synthetic oligonucleotide probes

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**Abstract.** Two synthetic oligonucleotide probe mixtures, whose sequences were inferred from two separate stretches of amino acids, one closer to the carboxy terminal and the other closer to the amino terminal, of ferredoxin I protein of *Azotobacter vinelandii,* were used to select ferredoxin I gene clones from a cosmid gene library of *Azotobacter vinelandii.* Restriction analysis revealed that 7 out of 10 selected clones were of the same type. All these clones were found to hybridize with *fixABCX* genes of *Rhizobium meliloti.*

**Keywords.** Ferredoxin; *Azotobacter vinelandii; fix* genes; nitrogenase; Fe–S cluster.

## Introduction

Many biological functions require auto-oxidizable low-potential ferredoxins and flavodoxins. The role of ferredoxins and flavodoxins in nitrogenase linked electron transport is well documented (Haaker, 1986). Ferredoxins are classified on the basis of their Fe–S clusters. *Azotobacter vinelandii,* an aerobic diazotroph, contains, in addition to ferredoxin II (Shethna *et al.,* 1968), which is a 2Fe–2S protein, another unique ferredoxin containing a 7Fe–7S cluster (Johnson *et al.,* 1987). This can split into 4Fe–S and 3Fe–3S clusters. The physiological significance of this unusual ferredoxin is not clear. It has been suggested that this protein might be required in aerobic nitrogen fixation in *A. vinelandii* (Kennedy and Toukdarian, 1987; Earl *et al.,* 1987). It has been found that the *fixA, B* and *C* genes of *Bradyrhizobium japonicum,* which are essential for nitrogen fixation in the free-living state, have counterparts in *A. vinelandii* (Gubler and Hennecke, 1986). Here we report the cloning of ferredoxin I gene of *A. vinelandii* by screening a gene library using synthetic oligonucleotide probes. Our observations suggest that the *fixABC* counterpart in *A. vinelandii* might be the gene encoding ferredoxin I and the gene(s) near it.

## Materials and methods

A gene library of *A. vinelandii* in the cosmid pHC79 was constructed. DNA from this library was transduced into *Escherichia coli* DH1 (Hanahan, 1983). Plasmid pDC2 containing *fix ABC* operon of *Rhizobium meliloti* was obtained from G. Ditta, University of California, San Diego, USA.

*E. coli* was grown in LB, as described by Miller (1972). Antibiotics used were: ampicillin, 50 $\mu$g/ml and tetracycline, 10 $\mu$g/ml.

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Plasmid isolation, restriction analysis, agarose gel electrophoresis and other techniques were as described in Maniatis *et al.* (1982).

## Results and discussion

### *Design and synthesis of the oligonucleotide probes and their labelling*

The amino-acid sequence of ferredoxin I from *A. vinelandii* has been published (Howard *et al.,* 1983). From this sequence two short regions, one close to the carboxy terminal and another close to the amino terminal, were selected for the synthesis of the corresponding oligonucleotide mixtures. Selection was based on minimum codon degeneracy in the oligonucleotides. The amino-acid sequence and deduced nucleotide sequence of each are presented in figure 1. These two 17-mer oligonucleotide probe mixtures were synthesized in the Pharmacia Gene Assembler using N,N-diisopropylphosphoramidite chemistry. After synthesis, the resin-bound oligomer was deprotected at the 5'–end. Oligonucleotides were cleaved from resin and deprotected with concentrated $NH_4.OH$ at 55°C for 16 h and separated from smaller molecular weight impurities on a Nap 10 column. The fragments were finally purified by FPLC using RPC column and 5–35% acetonitrile gradient in 0·1 M triethyl ammonium acetate buffer (pH 7). Synthesis was done on 0·2 $\mu$M scale with average coupling efficiency of 96 ± 1% in each step. These were 5'–end-labelled with [$\gamma$-$^{32}$P]-ATP (New England Nuclear, USA, 3000 Ci/mmol) using polynucleotide kinase following the instructions of the supplier. Colonies containing the recombinant plasmids of the library were grown at a density of 1000–5000 per Petri plate and transferred onto nitrocellulose membrane (Schleicher and Schuell, BA85). The cells were lysed and the DNA denatured and hybridized to the labelled probes according to Mason and Williams (1985). Ten of the colonies that gave positive signal with both probes were purified. Plasmids were isolated from cells grown out of these colonies and digested with restriction endonucleases *Bgl* II, *Eco* RI and *Pst* I (figure 2). The clones (40–45 kb) were found to be of 4 kinds, and 7 clones were of one kind.

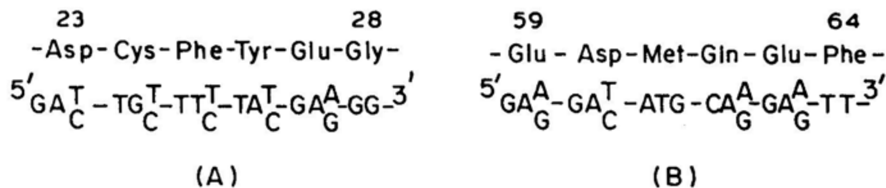


**Figure 1.** Sequences of two stretches of amino acids of ferredoxin 1 from *A. vinelandii* and the sequences of the oligonucleotide probe mixtures that have been synthesized. A. Sequence near the amino-terminal end. **B.** Sequence near the carboxy-terminal end.

### *Homology of DNA in the cosmid clones with fixABC genes*

A ~ 4·5 kb *Hin* dIII fragment from the plasmid pDC2 (Ditta, G., unpublished results; also see Earl *et al.,* 1987) containing the *fix ABC* genes of *R. meliloti* was isolated from a low-melting-agarose gel according to Maniatis *et al.* (1982), and nick-translated as

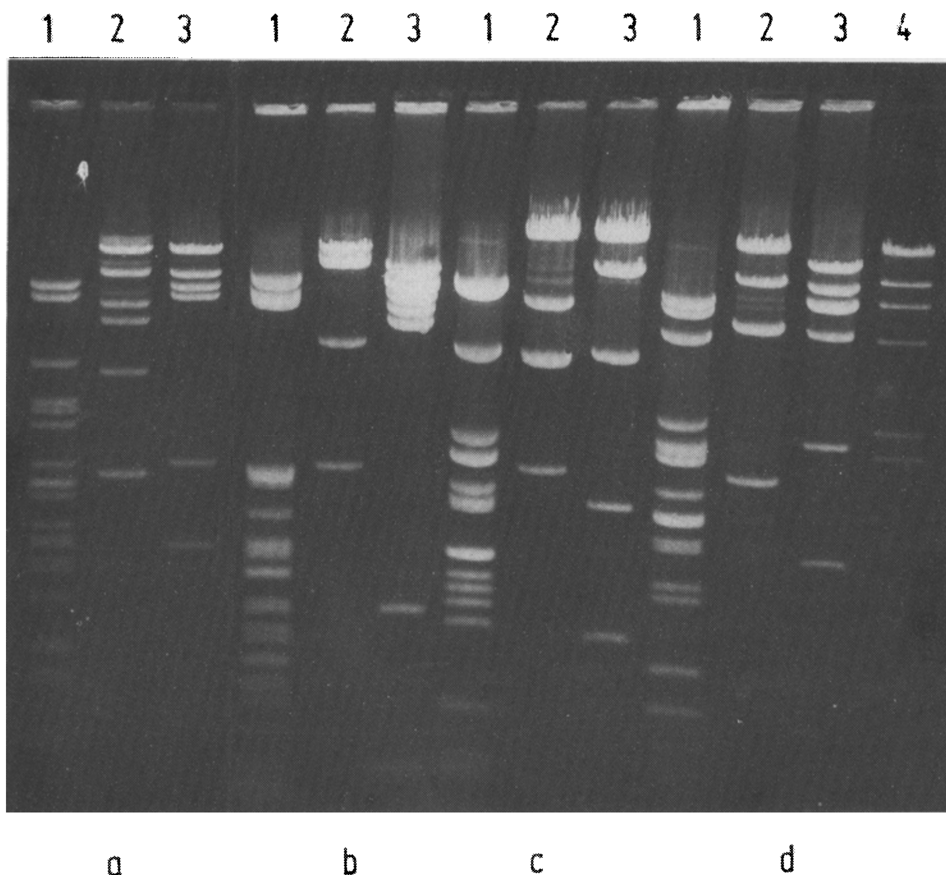


**Figure 2**. Restriction analysis of DNA from cosmid clones hybridizing to oligonucleotide probes, a, b, c and d are the 4 types of clones. Type c represents 7 cosmid clones, while a, b and d represent one clone each. Lane 1, DNA digested with *Pst* I; lane 2, DNA digested with *Bgl* II; lane 3, DNA digested with *Eco* RI; lane 4, lambda DNA digested with *Hin* dIII (size marker).

described by Rigby *et al.* (1977). DNA transfer onto nitrocellulose paper, hybridization, washing, etc. were carried out as described by Southern (1975) and Maniatis *et al.* (1982). *Eco* RI-digested DNA from all 4 types of clones was electrophoresed in agarose gel and transferred onto cellulose nitrate membrane according to Southern (1975). Hybridization of the DNA bands with labelled *fixABC* probe was then carried out according to Maniatis *et al.* (1982). Hybridization conditions were of high stringency. Hybridization was carried out in 6XSSC, 5XDenhardt, 0·5% SDS at 65°C overnight. The first washing was with 2XSSC, 0·1% SDS for 15 min at 25°C, and the final washing was with 0·lXSSC, 0·5% SDS for 2 h at 65°C. All 4 cosmid clones hybridized to this probe (figure 3). Gubler and Hennecke (1986) have reported a ~ 7 kb *Eco* RI fragment from genomic DNA of *A. vinelandii* hybridizing *to fixA, B* and *C* probe from *B. japonicum.* In two types of cosmids one band corresponded to this size, while in the other two it was different. This is probably because in these latter two clones the hybridizing region was next to

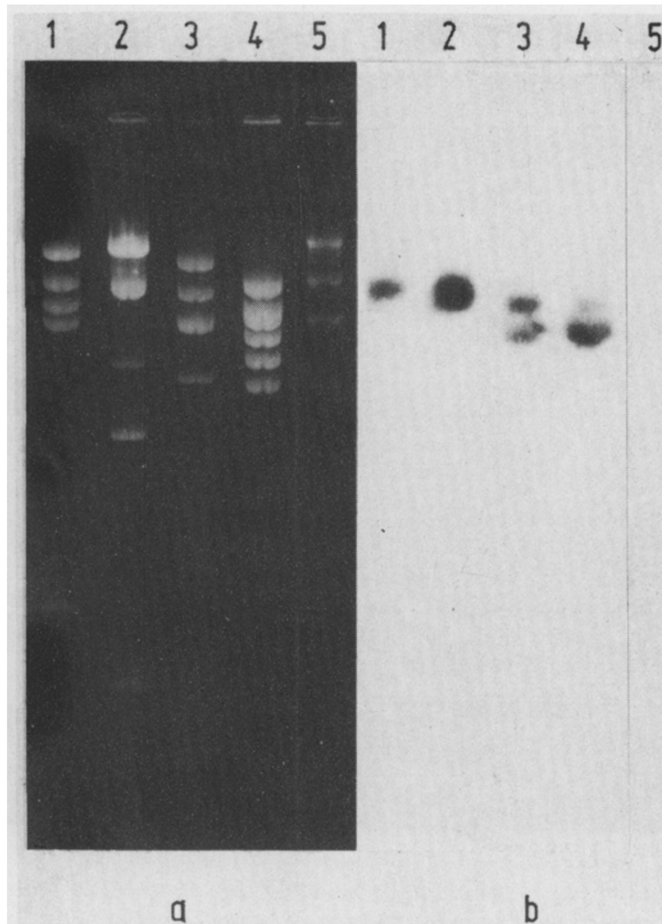


**Figure 3.** Hybridization of *fixABCX* probe from *R. meliloti* with DNA from the cosmid clones containing ferredoxin I-like sequences, **a**. Lane 1, *Eco*RI digested DNA from type a clone; lane 2, *Eco*RI digested DNA from type c clone; lane 3, *Eco*RI digested DNA from type d clone; lane 4, *Eco*RI digested DNA from type b clone; lane 5, lambda DNA digested with *Hin* dIII as size marker and negative control, **b**. Autoradiogram after Southern blot and hybridization.

the vector (this region also had ampicillin resistance). In the other two clones, one more band lighted up. We do not know whether it was due to some rearrangements or was because of a new region homologous to these genes. Recently, it has been reported that *fix ABC* operon of *R. meliloti* has another gene *fixX* (Earl *et al.,* 1987). Nucleotide sequence and predicted amino-acid sequence of this gene were found to be more than 60% homologous with the ferredoxin I sequence. Since a part of the *fixX* gene is also contained in the probe we have used, the hybridizing region in the *A. vinelandii* cosmid clones is also likely to contain the ferredoxin I gene. It is possible that there are other genes adjacent to the ferredoxin I gene which are involved in the protection of the nitrogenase system *in vivo.* Cloning of these genes unique to aerobic diazotrophs should lead to their further characterization and elucidation of the mechanism of protection from oxygen and the understanding of

the physiological significance of the unusual clustering of Fe–S centres in ferredoxin I.

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