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Conservation of a Pseudomonad-like Hydrocarbon Degradative Ferredoxin Oxygenase Complex Involved in Rhizopine Catabolism in *Sinorhizobium meliloti* and *Rhizobium leguminosarum* bv. *viciae*

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Abstract

In *Sinorhizobium meliloti* the *mocCABR* genes have previously been shown to be required for rhizopine (3-*O*-methyl-*scyllo*-inosamine, 3-*O*-M*S*I) catabolism. We show that the *mocDE(F)* gene cluster is also needed. MocDE(F), which is involved in the catabolism of 3-*O*-M*S*I to its demethylated form *scyllo*-inosamine (*S*I) has homology to components that would comprise a ferredoxin-oxygenase system. The *mocCABRDE*(*F*) suite of genes is required for 3-*O*-M*S*I catabolism in both *S. meliloti* and *R. leguminosarum* bv. *viciae*. However, *S*I catabolism in *S. meliloti* requires *mocCABR*, whereas only *mocCA* are required for its catabolism in *R. leguminosarum* suggesting the two species require different chromosomal genes which act in concert with *moc* genes for the catabolism of rhizopine.

The rhizobia (*Rhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium*) form symbiotic associations with legume plants reducing gaseous dinitrogen to ammonium ions that are readily assimilated by plants. This process occurs in nodules on plant roots. The rhizopine 3-*O*-methyl-*scyllo*-inosamine (3-*O*-M*S*I) is important in intra-species rhizobial competition for nodulation (Gordon *et al.,* 1996; Heinrich *et al.,* 1999). Rhizopines are produced in bacteroids within nodules by certain members of *Sinorhizobium meliloti* and *Rhizobium leguminosarum* bv. *viciae*. They are novel compounds in that only the strain which can induce rhizopine synthesis in the nodule can catabolize it in the free-living bacteria (Murphy and Saint 1992; Murphy *et al.,* 1995; Dessaux *et al.,* 1998). The genes for rhizopine synthesis and catabolism have been isolated and are termed *mos* and *moc* genes respectively (Murphy *et al.,* 1987; Murphy *et al.,* 1993).

Previously, the *mocCABR* gene cluster has been shown to be required for 3-*O*-M*S*I catabolism in *S. meliloti* strain L5-30 (Rossbach *et al*., 1994). Homologies between the deduced proteins from this region and proteins in data bases suggest that MocA and MocC most likely have catabolic roles and MocB and MocR are probably involved in transport and regulation respectively (Rossbach *et al*., 1994).

More recently, we have shown in *R. leguminosarum* bv. *viciae* strain 1a that besides *mocCABR* two additional genes (*mocD* and *mocE*) and a likely third (*mocF*) are also required for 3-*O*-M*S*I catabolism. These genes are involved in the conversion of 3-*O*-M*S*I to its demethylated form, *scyllo*-inosamine (*S*I), in the rhizopine degradative pathway (Bahar *et al.,* 1998).

We have used a combination of deletion/complementation studies, Tn*5*-B20 mutagenesis and DNA sequencing to determine whether these additional genes are also required for 3-*O*-M*S*I catabolism in *S. meliloti*. These studies indicate that situated immediately downstream of *mocCABR* are the *mocDE*(*F*) genes that are also required for 3-*O*-M*S*I catabolism (Figure 1). When *mocEF* and part of *mocD* are removed from the *moc* cluster *S*I (the demethylated form of 3-*O*-M*S*I) but not 3-*O*-M*S*I is catabolised (Figure 1). The *mocDE*(*F*) genes are therefore involved in demethylation of 3-*O*-M*S*I to *S*I.

In *R. leguminosarum* bv. *viciae* strain 1a the MocDEF proteins were proposed to form a ferredoxin oxygenase complex. This oxygenase system would use the ferredoxin reductase (MocF) to transfer electrons from NADH to the ferredoxin (MocE). Electrons would then be transferred to the oxygenase (MocD) to convert the methyl group of 3-*O*-M*S*I to a hydroxyl group thus forming *S*I (Bahar *et al.,* 1998). This model is similar to that proposed for the initial steps in the breakdown of xylene by *Pseudomonas putida* (Suzuki *et al.,* 1991).

There is extensive homology (81%, 82%, 71% identity at the amino acid level, respectively) between MocD, E and F in *R. leguminosarum* bv. *viciae* 1a and *S. meliloti.* Importantly, consensus sequences present in proteins forming a ferredoxin oxygenase complex are present. *S. meliloti* MocD, the proposed oxidative component, possesses three histidine boxes (His-X-X-X-His-31-His-X-X-His-His-145-His-X-X-His-His) located in hydrophobic regions of the MocD protein which is characteristic of oxidative enzymes like xylene-monooxygenase (Suzuki *et al.,* 1991) and alkane hydroxylase (Kok *et al*., 1989). MocE, a small protein of 106 amino acids has the conserved sequence C-X-H-X15-17-C-X2-H found in Rieske-like

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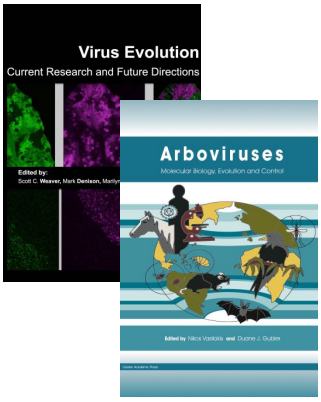

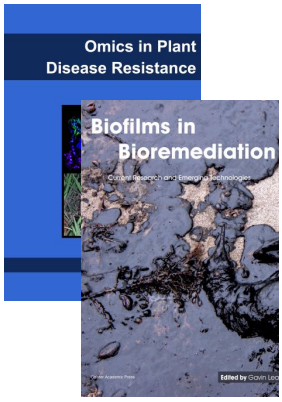

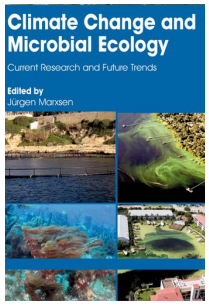

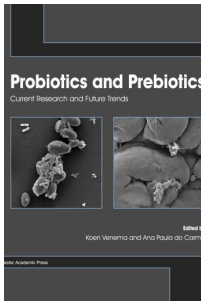

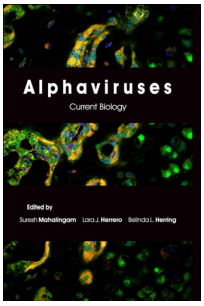

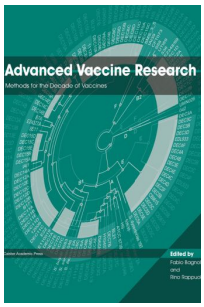

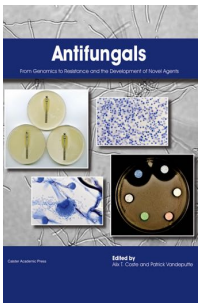

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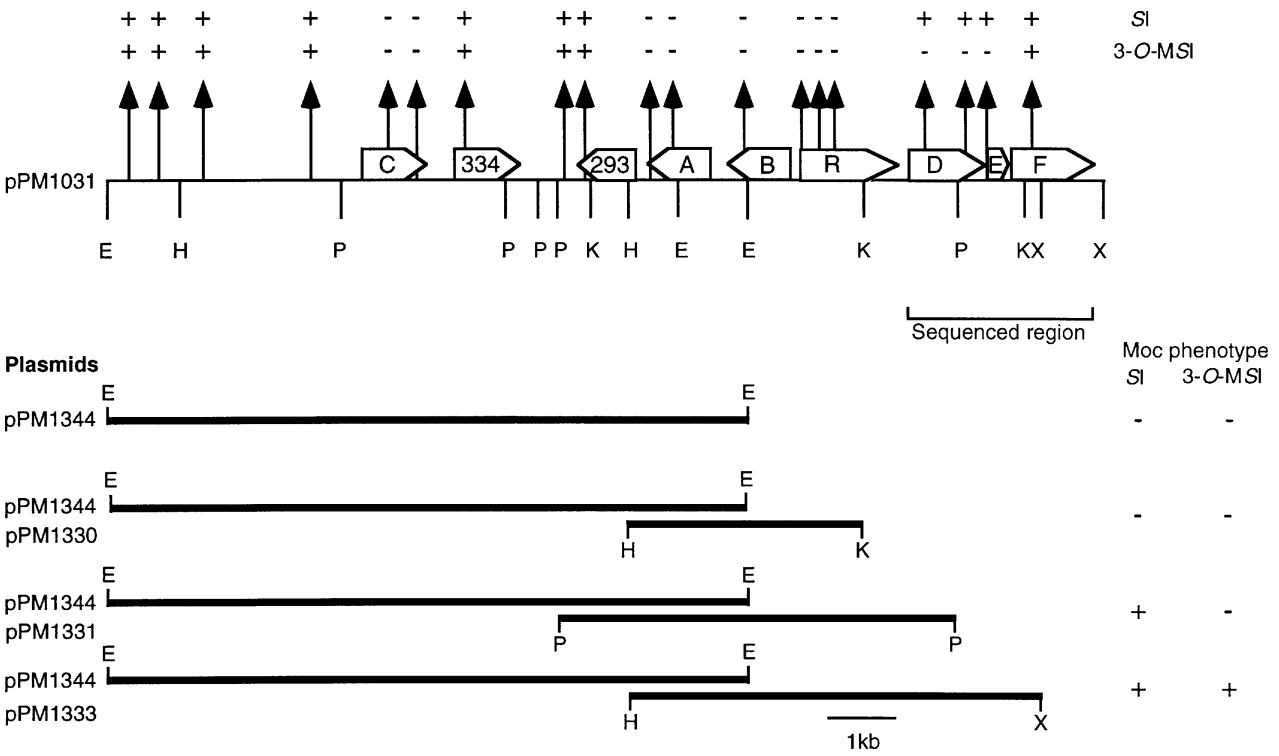

Figure 1. Structure of the rhizopine *moc* cluster in *S. meliloti* strain L5-30.
The region of the diagram showing *mocC,* ORF334, ORF293, *mocA*, *mocB* and *mocR* is based on data from Rossbach *et al.,* 1994. Sites of Tn*5*-B20 insertion as well as *S*I and 3-*O*-M*S*I catabolic phenotype over the whole region shown in pPM1031 were determined in this study. For this purpose the plasmid pPM1031 was mutagenized with the transposon Tn*5*-B20 (Simon *et al*., 1989) as described previously (Bahar *et al.,* 1998). The narrow vertical arrows indicate the location of Tn*5*-B20 inserts. Above these the catabolic phenotypes for 3-*O*-M*S*I and *S*I are indicated by "+" (catabolism) and "-" (non-catabolism). Plasmids used for complementation studies are shown by lines below the map. Plasmids pPM1031 and pPM1344 contain fragments cloned into pLAFR1 (Inc P-1) and plasmids pPM1330, pPM1331, pPM1333 contain fragments cloned into pUCD2608 (Inc W). The catabolic phenotypes (+, -) for 3-*O*-M*S*I and *S*I are indicated to the right. Catabolism tests with *S*I and 3-*O*-M*S*I were performed after transferring mutated pPM1031 or clones described above into *S. meliloti* strain Rm1021. Transfer was by mating using the helper plasmid pRK2013 (Bahar *et al.,* 1998). Rm1021 is a wildtype Moc$^-$, Mos$^-$ *S. meliloti* strain (Meade *et al.,* 1982). The region sequenced in this study is indicated by a thin line. The GenBank nucleotide accession number for the *S. meliloti* strain L5-30 *mocDEF* gene cluster is AF076471. Open reading frames are indicated by large open arrows. Only restriction enzyme sites referred to are shown. E, *Eco*R I; H, *Hin*d III; K, *Kpn* I; P, *Pst* I; X, *Xho* I.

ferredoxins involved in hydrocarbon breakdown (Morrice *et al.,* 1988; Tan *et al.,* 1993). Although MocF is not essential for 3-*O*-M*S*I catabolism it contains two copies of the consensus sequence G-X-G-X2-G-X3-A-X6-G characteristic of NAD-dependent ferredoxin reductase components which interact with ferredoxins in ring hydroxylating oxygenase reactions in hydrocarbon degradation (Zylstra and Gibson, 1989). It is possible the non-essential nature of MocF can be explained by it being substituted by another ferredoxin reductase elsewhere in the genome.

The results presented here differ from those described by Rossbach *et al*. (1994) who showed that *mocCABR* were sufficient for 3-*O*-M*S*I catabolism. As our study indicates that these genes only catabolised *S*I it is possible that *S*I and not 3-*O*-M*S*I was utilized in the Rossbach study. These compounds would not be distinguishable using the paper electrophoretic conditions reported.

In *S. meliloti mocCABR* are required for *S*I catabolism whereas in *R. leguminosarum mocCA* are sufficient. Hence, as *mocB* is a putative transport protein (Rossbach *et al*., 1994), *R. leguminosarum*, but not *S. meliloti*, likely has a gene outside of the *moc* cluster which can substitute for *mocB* to transport *S*I into the cells. Furthermore, this implies that the *S. meliloti mocR* gene is involved in regulation of both the demethylation of 3-*O*-M*S*I to *S*I and then the further catabolism of *S*I whereas in *R. leguminosarum* it only functions in regulation of the demethylation step. This suggests that in *R. leguminosarum*, the further catabolism of *S*I may be regulated by another gene, or alternatively, genes other than those in the *moc* cluster are involved in the catabolism of 3-*O*-M*S*I and these may be different in *R. leguminosarum* and *S. meliloti*.

In conclusion this study has shown that the *moc* cluster (*mocCABRDE(F)*) required for 3-*O*-M*S*I catabolism is conserved in *S. meliloti* strain L5-30 and *R. leguminosarum* bv. *viciae* strain 1a. In what are quite distantly related species (Young, 1996) the *moc* genes are highly homologous. The breakdown of 3-*O*-M*S*I proceeds via *S*I and appears to involve a *mocDEF* encoded Reiske-like ferredoxin oxygenase system similar to those found in hydrocarbon catabolism in pseudomonads. However, since *R. leguminosarum* does not require all of the *moc* genes that *S. meliloti* requires to catabolize *S*I it suggests genes

outside of the *moc* cluster, which act in concert with *moc* genes, are different in the two species. The rhizopine catabolic genes appear to have an adaptive role utilizing available functions in their host species to enable rhizopine catabolism to proceed.

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