A Ti Plasmid-Encoded Enzyme Required for Degradation of Mannopine Is Functionally Homologous to the T-Region-Encoded Enzyme Required for Synthesis of This Opine in Crown Gall Tumors

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Received 16 January 1996/Accepted 28 March 1996

The mocC gene encoded by the octopine/mannityl opine-type Ti plasmid pTi5955 is related at the nucleotide sequence level to mas1 encoded by the T region of this plasmid. While Mas1 is required for the synthesis of mannopine (MOP) by crown gall tumor cells, MocC is essential for the utilization of MOP by Agrobacterium spp. A cosmids clone of pTi5955, pYDH208, encodes mocC and confers the utilization of MOP on strain NT1 and on strain UIA5, a derivative of NT1 lacking the 450-kb cryptic plasmid pATC58. NT1 or UIA5 harboring pYDH208 with an insertion mutation in mocC failed to utilize MOP as the sole carbon source. Plasmid pSa-C, which encodes only mocC, complemented this mutation in both strains. This plasmid also was sufficient to confer utilization of MOP on NT1 but not on UIA5. Computer analysis showed that MocC is related at the amino acid sequence level to members of the short-chain alcohol dehydrogenase family of oxidoreductases.

Lysates prepared from Escherichia coli cells expressing mocC contained an enzymatic activity that oxidizes MOP to deoxyfructosyl glutamine (sanhopine [SOP]) in the presence of NAD⁺. The reaction catalyzed by the MOP oxidoreductase is reversible; in the presence of NADH, the enzyme reduced SOP to MOP. The apparent Km values of the enzyme for MOP and SOP were 6.3 and 1.2 mM, respectively. Among analogs of MOP tested, only N1-(1-deoxy-o-mannityl)-L-glutamine and N1-(1-deoxy-o-mannityl)-L-asparagine served as substrates for MOP oxidoreductase. These results indicate that mocC encodes an oxidoreductase that, as an oxidase, is essential for the catabolism of MOP. The reductase activity of this enzyme is precisely the reaction ascribed to its T-region-encoded homolog, Mas1, which is responsible for biosynthesis of mannopine in crown gall tumors.

Agrobacterium tumefaciens and Agrobacterium rhizogenes cause crown gall and hairy root diseases in dicotyledonous plants. The genes for tumorigenicity are carried on the T regions of large epigenetic elements called Ti and Ri plasmids present in A. tumefaciens and A. rhizogenes, respectively. During infection, the T-region DNA is transferred from the bacteria to the plant cell, in which it becomes stably integrated into the nuclear genome. The T-DNA also encodes genes for the synthesis of novel low-molecular-weight compounds, called opines, in the transformed plant cells. The opines produced and secreted by the neoplastically transformed cells are utilisable as sole carbon and energy sources by the agrobacteria that induced the tumor. The genes for the utilization of the opines by the bacteria also are encoded by the Ti plasmid, but they are located outside the T-DNA region (6, 29, 31).

The mannityl opines are among the more than 20 opines grouped into nine families identified to date that are associated with the Agrobacterium pl-plant interaction (6). Four compounds belong to this family: mannopine (MOP) and mannopinic acid (MOA) are imine conjugates of mannose and glutamine and of mannose and glutamic acid, respectively. Agropine (AGR) and agropinic acid are cyclized derivatives of MOP. The biosynthesis of these opines by transformed plant cells requires three genes encoded by the Tregion of octopine- and agropine-type Ti plasmids (2, 8, 28). Ellis et al. (8) proposed a pathway in which mas2 encodes a conjugase that condenses glucose and glutamine or glutamic acid to yield deoxyfructosyl glutamine (also called sanhopine [SOP]) or deoxyfructosyl glutamic acid, respectively. These intermediates then are believed to be reduced to MOP and MOA by a reductase encoded by mas1. Finally, MOP is lactonized to AGR by the product of mas1.

While only three genes are required for the synthesis of the mannityl opines by the tumors, the genes for catabolism of these substrates by the bacteria occupy a contiguous 45-kb region of the Ti plasmid (7). A 21-kb region of pTi5955 contained on the cosmids clone pYDH208 confers on strain NT1, a Ti plasmid-cured derivative of strain C58, the ability to utilize MOP and AGR as sole carbon sources (12). Molecular genetic studies of this clone indicated that four of the genes involved in the catabolism of MOP and AGR are homologs of the mas genes (12, 22). The gene ageA encodes an enzyme, called catabolic MOP cyclase (14), that lactonizes MOP to AGR. This enzyme is a structural and functional homolog of Mas0 (12, 14). mocD, which encodes an enzyme of unknown activity, is a homolog of mas2, which is believed to encode the biosynthetic conjugase. Interestingly, the mocE and mocC genes have homologs with the 5′ half and the 3′ half, respectively, of mas1 (22). Considering these similarities, we have proposed that the mas genes and the moc genes evolved from a common
NH₄)₂SO₄ and mannitol or MOP at 5 mM was used to grow Agrobacterium tumefaciens. AT minimal medium supplemented with 0.15% Kpn Isites of the two plasmids. Plasmid pSa-C was constructed by ligating the entire pKS-C from pBSS14 into the vector (Fig. 1), and Escherichia coli producing pKS-C. In this plasmid, pSaB4 (22) into the vector (Fig. 1) (13). Plasmid pBSS14 was constructed by ligating the 4.1-kb pKS-C gene with its own putative Shine-Dalgarno (S.D.) sequence is cloned behind the lac promoter of pUC19. See text for details.

FIG. 1. Genetic organization of the moc region on pYDH208. The positions of Tn5 insertions and the abilities of strains NT1 and UIA5 carrying each of the mutant derivatives to utilize MOP as the sole source of carbon are indicated: −, no growth; +, good growth but slower than wild-type growth; ++, wild-type growth; ++++, faster than wild-type growth. The open arrows indicate genes defined by mutation and DNA sequence analysis (22) that are associated with catabolism of MOP and AGR. Mutation 256, in which Tn5 is inserted in mocC, is boxed. The structure of pKS-C is shown at the bottom; the mocC gene with its own putative Shine-Dalgarno (S.D.) sequence is cloned behind the lac promoter of pUC19. See text for details.

MATERIALS AND METHODS

Bacterial strains and plasmids. \textit{A. tumefaciens} NT1 is a Ti plasmid-cured derivative of C58 (13). Strain UIA5 is a derivative of C58 that lacks both the Ti plasmid and the 450-kb cryptic megaplasmid pATc58 (22, 27). Cosmid clone pYDH208::Tn5 256 carries a Tn5 insertion in the mocC gene (Fig. 1) (13). Plasmid pBSS14 was constructed by ligating the 4.1-kb Smal fragment 14a from pSaB4 (22) into an EcoRV site of pBlueScript SK−. A 1.6-kb SplI-KpnI fragment from pBSS14 was recloned into the high-copy-number vector pUC19 to produce pKS-C. In this plasmid, mocC is transcribable from the lac promoter of the vector (Fig. 1), and \textit{Escherichia coli} DH5α carrying this plasmid expresses the MocC protein (22). Plasmid pSa-C was constructed by ligating the entire pKS-C plasmid into the broad-host-range IncW vector pSa4AH (11), using the unique KpnI sites of the two plasmids.

Culture media and chemicals. Nutrient broth (Difco) and L-broth (LB; Gibco) were used as the rich media. AT minimal medium supplemented with 0.15% (NH₄)₂SO₄ (7) and mannitol or MOP at 5 mM was used to grow Agrobacterium strains. Noble agar (final concentration, 1.8%); Difco) was used to solidify the minimal medium. \textit{E. coli} strains were grown at 37°C, and \textit{Agrobacterium} strains were grown at 28°C. MOP was synthesized as described previously (25). Stock solutions were prepared in double-distilled water at 100 mM, sterilized by filtration, and kept frozen at −20°C. SOP and the MOP analogs listed in Table 1 were prepared as described previously (5).

MOP utilization studies. Growth was assessed by visual inspection of colonies on solid media at daily intervals over a 1-week period. Growth was recorded on the basis of comparisons with appropriate positive and negative controls, as follows: −, no growth; +, good growth but less than wild-type growth; ++, wild-type growth; or ++++, faster than wild-type growth. Opine utilization was verified by analyzing liquid culture supernatants for disappearance of the substrate as determined by high-voltage paper electrophoresis (HVPE) (25).

Preparation of cell extracts. An overnight culture of \textit{E. coli} DH5α(pKS-C) was inoculated into 50 ml of LB containing ampicillin. The cells were grown at 37°C with shaking until growth reached late exponential phase (80 to 100 Klett units). Cells were harvested by centrifugation (10 min, 7,000 g) and washed twice with 0.9% NaCl. Cells were suspended in 5 ml of 0.1 M Tris-HCl (pH 8.6) and disrupted by sonication (Branson Sonifier 450) on ice for two 5-min periods, separated by a 1-min cooling interval. Cell debris was removed by centrifugation (10 min, 20,000 g, 4°C). The supernatant was diluted with 5 ml of 0.1 M Tris-HCl (pH 8.6), and the diluted cell extract, containing approximately 650 μg of total protein per ml, was used for all enzyme assays. Protein

### TABLE 1. Analogos of mannopine used in this study

<table>
<thead>
<tr>
<th>MOP analog</th>
<th>Alternative name or abbreviation used in this report</th>
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<tbody>
<tr>
<td>N-1-(1-deoxy-D-glucityl)-L-glutamine</td>
<td>Glucopine</td>
</tr>
<tr>
<td>N-1-(1-deoxy-D-galactityl)-L-glutamine</td>
<td>Galactopine</td>
</tr>
<tr>
<td>N-1-(1-deoxy-D-arabinityl)-L-glutamine</td>
<td>d-Lyx-Gln</td>
</tr>
<tr>
<td>N-1-(1-deoxy-D-ribofuranosyl)-L-glutamine</td>
<td>MOA</td>
</tr>
<tr>
<td>N-1-(1-deoxy-D-erythityl)-L-glutamine</td>
<td>d-Man-Val</td>
</tr>
<tr>
<td>N-1-(1-deoxy-D-lyxosyl)-L-glutamine</td>
<td>d-Man-Asn</td>
</tr>
<tr>
<td>N-1-(1-deoxy-D-mannoxylyl)-L-glutamine</td>
<td>d-Man-Met</td>
</tr>
</tbody>
</table>

* A natural member of the mannotyl opine family.
The nucleotide sequence of MocC is available in GenBank under accession number U19620. It is deduced amino acid sequences translatable from these three quencher motifs common in the deduced amino acid sequences of MocC and its software package from Genetics Computer Group Inc. (Madison, Wis.).

**Definition of unit and specific activity.** One unit of MOP oxidoreductase was defined as the amount of enzymatic activity that catalyzes the reduction of 1 μmol of NAD⁺ (when MOP was used as the substrate) or the oxidation of 1 μmol of NADH (when SOP was used as the substrate) per minute. Specific activity was defined as micromoles of NADH oxidized or of NAD⁺ reduced per minute per milligram of total protein present in the assay.

**Kinetic measurements.** Apparent Kᵣ values were determined from initial reaction rates measured at substrate concentrations ranging from 1 to 7 mM for MOP and from 0.5 to 5 mM for SOP.

**HYPE.** Opines were resolved by HYPE in an acetic acid-formic acid buffer (pH 1.8) (25) or in 0.1 M ammonium bicarbonate buffer (pH 9.6) as described previously (8). Opines were detected by the alkaline silver nitrate staining technique or by staining with 2,3,5-triphenyltetrazolium as described previously (8).

**Alignment of deduced amino acid sequences.** The amino acid sequence of the protein translatable from mocC (22) was compared with sequences in the GenBank database by using the BLAST search program (1). Alignments of amino acid sequences were performed by using the Gap program (24) in the GCG software package from Genetics Computer Group Inc. (Madison, Wis.). Sequence motifs common in the deduced amino acid sequences of MocC and its homologs were identified by using the Motifs program in the GCG software package.

**Nucleotide sequence accession number.** The nucleotide sequence of mocC was deposited in GenBank under accession number U19620. Its deduced amino acid sequence is reported in reference 22.

### RESULTS

Some moc mutations are complemented by functions encoded in strain NT1. Plasmid pYDH208, a cosmide clone with a 21-kb insert from pTi55955, confers the utilization of MOP as the sole carbon source on strain NT1. This clone also allows strain UIA5 to grow with MOP as the sole carbon source (Table 2).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth on MOP*</th>
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<tbody>
<tr>
<td>NT1</td>
<td>+</td>
</tr>
<tr>
<td>UIA5</td>
<td>+</td>
</tr>
<tr>
<td>UIA5(pYDH208)</td>
<td>+</td>
</tr>
<tr>
<td>NT1(pYDH208)</td>
<td>-</td>
</tr>
<tr>
<td>UIA5(pSa-C)</td>
<td>+</td>
</tr>
<tr>
<td>NT1(pSa-C)</td>
<td>+</td>
</tr>
<tr>
<td>UIA5(pYDH208:256,pSa-C)</td>
<td>+</td>
</tr>
<tr>
<td>NT1(pYDH208:256,pSa-C)</td>
<td>+</td>
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</table>

* Symbols: --, no growth; +, wild-type growth.

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stained with alkaline silver nitrate but not with 2,3,5-triphenyl tetrazolium chloride (data not shown). These results clearly indicate that MocC is an oxidoreductase that reversibly converts MOP to SOP.

On the basis of these results, we developed a quantitative enzyme assay in which the activity of MOP oxidoreductase can be assessed by measuring the reduction of NAD\(^+\) (Fig. 4B). At pH values of 8.8, 8.0, and 7.0, the oxidoreductase showed 96, 39, and 6%, respectively, of the maximum activity. At pH 9.0, the enzyme showed no activity. Therefore, we performed all subsequent enzyme assays at pH 8.6.

**MOP oxidoreductase is a cytoplasmic enzyme.** To determine if MOP oxidoreductase is cytoplasmic or membrane associated, we assayed the soluble and the washed particulate fractions, collected following sonication and centrifugation, for NAD\(^+\) reductase activity with MOP as the substrate. While the soluble fraction contained high amounts of activity, the fraction containing insoluble cell debris did not detectably reduce the cofactor in the presence or absence of the opine substrate (data not shown).

**The reaction catalyzed by MOP oxidoreductase is reversible.** We measured the reversibility of the MOP oxidoreductase activity quantitatively as follows. When the oxidation of 1 \(\mu\)mol of MOP to SOP by the enzyme had almost reached the maximum level, the reaction was supplemented with 1 \(\mu\)mol of SOP. Under these conditions, the SOP was rapidly reduced to MOP by using NADH which had been generated from NAD\(^+\) by the oxidation of MOP to SOP. The reaction eventually reached an equilibrium with the NADH level at about 14% of its maximum level obtained during the oxidation of MOP to SOP (Fig. 5). These results indicate that the activity of MOP oxidoreductase is freely reversible.

**Reduction of SOP to MOP is the dominant activity of MOP oxidoreductase.** Our qualitative analysis by HVPE suggested that the reduction of SOP to MOP by MOP oxidoreductase occurs at a rate faster than that of the reverse reaction (Fig. 3). Our kinetic analysis confirmed this: the \(V_{\text{max}}\) for the reduction of MOP to SOP was greater than that of the oxidation reaction at all substrate concentrations tested (data not shown). Furthermore, under the conditions tested, the enzyme exhibited an apparent \(K_m\) for SOP of approximately 1.2 mM, while that for MOP was approximately 6.3 mM (Fig. 6).

**Substrate specificity of MocC.** We tested various MOP analogs with alternate sugar or alternate amino acid residues as substrates for MOP oxidoreductase (Fig. 7). Among the sugar analogs tested, only D-Lyx-Gln (see Table 1 for abbreviations) was oxidized by the enzyme at a detectable level. However, the reaction rate with this analog as a substrate is only 30% of that stained with alkaline silver nitrate (8).

![FIG. 2. Alignment of the deduced amino acid sequences of mocC and its homologs.](image)

![FIG. 3. Oxidation of MOP to a new product catalyzed by MocC protein and NAD\(^+\).](image)
observed with MOP. Among amino acid analogs tested, d-Man-Asn was oxidized efficiently by MOP oxidoreductase. The other three amino acid analogs tested gave no or barely detectable levels of oxidation by the enzyme. Among these, MOA, a natural member of the mannitylpine family, was not oxidized by MOP oxidoreductase at a significant rate under the conditions tested.

**DISCUSSION**

Ellis et al. (8) proposed that in crown gall tumors, the T-DNA-encoded Mas1 catalyzes the reduction of SOP to MOP. The deduced amino acid sequence of MocC shows homology with various oxidoreductases of the short-chain alcohol dehydrogenase family (Fig. 2). As reported previously, the sequence of MocC also shows homology with the deduced amino acid sequence of the carboxyl-terminal half of Mas1 encoded by the T region of pTi15955 (22). Furthermore, the sequences of both MocC and Mas1 contain a motif which is conserved in all NAD\(^+\)-dependent oxidoreductases (9). Conserved tyrosine and lysine residues in this motif, which is essential for the enzymatic activity of the short-chain alcohol dehydrogenase family of oxidoreductases (9), is present in both MocC and Mas1 (Fig. 2). This observation suggested to us that MocC is an oxidoreductase that can oxidize MOP to SOP by using NAD\(^+\) as an electron acceptor. In this study, we confirmed this prediction biochemically. A cell-free lysate prepared from E. coli DH5\(\alpha\) expressing mocC reduced MOP to SOP, using NAD\(^+\) as an electron acceptor. That MOP oxidoreductase localizes to the cytoplasmic fraction contrasts with octopine oxidase, also encoded by pTi15955 (20). This latter enzyme is strongly membrane associated and does not require soluble cofactors. Moreover, its two subunits, OoxA and OoxB, are not related to MOP oxidoreductase at the amino acid sequence level (results not shown). However, the location of MOP oxidoreductase is consistent with its inclusion in the family of short-chain alcohol dehydrogenases, all of which are cytoplasmic enzymes (4).

mocC not only is essential but also is sufficient to confer the utilization of MOP on *A. tumefaciens* NT1. Considering that the genes for the utilization of the mannitylpines span almost one-quarter (approximately 45 kb) of pTi15955 (7), it was surprising that this single gene can confer the utilization of...
MOP. This observation strongly suggests that the genome of NT1 encodes functions that are responsible for the degradation of SOP produced by the oxidation of MOP by MOP oxidoreductase. Moreover, the genome of NT1 must encode a transport system that recognizes MOP. Vaudequin-Dransart et al. (30) recently showed that SOP is degraded by the Ti plasmidless strain NT1. They also showed that a strain lacking the cryptic plasmid pAtC58 could not utilize SOP, suggesting that catabolism of this opine requires genes encoded by the megaplasmid. Consistent with this finding, our work shows that insertions in mocD, encoding an enzyme that we believe splits SOP, or in the transport system for MOP or AGR encoded by pYDH208 abolish the ability of UIA5 to utilize SOP, suggesting that NT1 harboring any of these mutants still utilizes the opine. These observations suggest that a SOP utilization system is encoded, at least in part, by the megaplasmid in NT1 and that this system suppresses the defects in the moc genes of the pYDH208 mutants. Presumably, the SOP produced by the oxidation of MOP by MOP oxidoreductase can be degraded by MocD encoded by the Ti plasmid or, in the absence of this gene, by functions encoded by pAtC58. Therefore, we regard MOP oxidoreductase as a key enzyme that can link the MOP degradation system encoded by the Ti plasmid with the SOP degradation system encoded elsewhere in the Agrobacterium genome.

It appears that in strain NT1(pKS-C), MOP is taken up through a transport system encoded by the megaplasmid. While NT1 harboring derivatives of pYDH208 with insertions in the regions encoding the MOP and AGR transport systems still utilized MOP as the sole carbon source, UIA5 harboring the same plasmids failed to grow with this opine (Fig. 1). We propose that pAtC58 encodes a transport system that recognizes both opines but is inducible only by SOP. In support of this view, while strain NT1 does not take up MOP at a detectable level (13), we recently isolated spontaneous mutants from this strain which can transport this opine quite well (21). No such mutants could be isolated from UIA5, suggesting that the cryptic plasmid is at least in part responsible for the transport system involved in MOP uptake by the NT1 mutant.

Considering that MOP oxidoreductase is essential for the catabolism of MOP, it is not clear why the bacterium employs an enzyme in which the reductase activity is more efficient than the oxidase activity. However, this is not without precedent in the mannityl opine system. In the case of the catabolic MOP oxidoreductase, the enzyme is essential for the degradation of the opine, and its structure is optimized for the reductase activity.

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**FIG. 6.** Lineweaver-Burk analyses of MOP oxidoreductase activity. Values of 1/V0 were determined for MOP (A) and SOP (B) as substrates. MOP was used at concentrations of from 1 to 7 mM, and SOP was used at concentrations of from 0.5 to 5 mM. At each concentration of substrate, the initial reaction rate was determined by measuring the rates of reduction of NAD+ or oxidation of NADH spectrophotometrically as described in Materials and Methods.

**FIG. 7.** Substrate specificity of MOP oxidoreductase. Cell extracts from E. coli DH5a(pKS-C) were incubated with MOP or its sugar or amino acid analogs in the presence of NAD+. The reduction of NAD+ to NADH was monitored spectrophotometrically as described in Materials and Methods. (A) Sugar analogs of MOP as substrates. Others, the remaining sugar analogs listed in Table 1. (B) Amino acid analogs of MOP as substrates.
cyclase, the lactonase activity of the enzyme is absolutely required for degradation of AGR. However, like MOP oxidoreductase, the reverse reaction, that is, the lactonization of MOP to AGR, is more efficient in vitro and in vivo (14, 15). It is possible that when presented with these opiines as substrates, manitylopine-utilizing Agrobacterium spp. convert SOP to MOP by using MOP oxidoreductase and further convert MOP to AGR by using the lactonizing activity of MOP cyclase. We propose that this constitutes a novel strategy whereby the bacterium sequesters a carbon source produced by the tumor that can be utilized by other competitors in the soil. The capacity to degrade Amadori compounds such as SOP has been found in other soil-associated microorganisms (10, 16–18). It is intriguing that both enzymes associated with catabolism of MOP and AGR exhibit reaction kinetics that favor the biosynthetic rather than catabolic conversions. It also is intriguing that the favored reaction direction is precisely that proposed by Ellis et al. (8) for the biosynthesis of these opiines in crown gall tumors catalyzed by the T-DNA homologs of these two enzymes. This finding is entirely consistent with our hypothesis that the genes encoding the catabolic and biosynthetic enzymes derived from a common ancestor (22).

MOP oxidoreductase shows a narrow substrate specificity. It appears that an amide group in the amino acid residue is required for the activity of the enzyme and that the conformation and the hydroxyl groups at the C-2′, C-3′, and C-4′ positions of the sugar residue are important for the activity. This specificity contrasts with that of MOP cyclase (15). Glucopine and galactopine, which are not oxidized by MOP oxidoreductase, are lactonized by MOP cyclase (15). However, d-Lyx-Gln and d-Man-Asn, which can be oxidized by MOP oxidoreductase, are not substrates for MOP cyclase. It is surprising that MOA, which is a natural member of the manitylopine family, cannot serve as a substrate either for MOP cyclase or, more importantly, for MOP oxidoreductase. Strain NT1(pYDH208) can utilize MOA when preinduced with MOP or AGR (13).

These observations led us to consider the possibility that MOA can be degraded by another pathway present in the bacterium. However, NT1 harboring pYDH208 with a transposon insertion in mocC cannot utilize this opine even following preinduction by MOP or AGR (13). This result suggests that MOP oxidoreductase is required by NT1(pYDH208) to utilize MOA. Given the in vitro substrate specificity of this enzyme, it remains to be determined how this strain can utilize MOA as a carbon source.

This study together with our work on MOP cyclase (12–15) supports our model, shown in Fig. 8, in which the pathway for catabolism of AGR and MOP in the bacteria is the reverse of the pathway for biosynthesis of these opiines in crown gall tumors (8). The finding that MOP oxidoreductase converts MOP to SOP led us to consider the link between the catabolic system for MOP encoded by octopine- and agropine-type Ti plasmids and that for SOP encoded by pAtC58. Inherent in this model is the redundancy of the SOP catabolism system. It is clear that the moc system contained on the Ti plasmid and its cosmid clone pYDH208 encodes the enzymes necessary for the degradation of SOP. Strain UIA5(pYDH208), which lacks pAtC58 and its associated SOP system, catabolizes MOP as well as does strain NT1(pYDH208). Yet strain NT1, which harbors the megaplasmid but lacks any moc functions, also catabolizes SOP. Future studies of the Moc system encoded by pTI15955 and the SOP utilization systems encoded by the Ti plasmid and by pAtC58 will provide more information on the relationship between these two gene systems and how they cooperate to promote the opine-driven interaction between Agrobacterium spp. and its host plant.

ACKNOWLEDGMENTS

We thank Paul D. Shaw and John Cronan, Jr., for helpful discussion on the enzymology of MOP oxidoreductase. We also thank Y. Dessaux and V. Vauduin-Dramsart for discussions on the relationship between SOP catabolism and MOP catabolism.
This work was supported in part by grants to S. K. Farrand from the Illinois Soybean Program Operating Board and the Biotechnology Research and Development Corporation.

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