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J. Bacteriol. **1997, 179(15):4831.**

JOURNAL OF BACTERIOLOGY, Aug. 1997, p. 4831–4840 Vol. 179, No. 15
0021-9193/97/$04.00+0

A T-DNA Gene Required for Agropine Biosynthesis by Transformed Plants Is Functionally and Evolutionarily Related to a Ti Plasmid Gene Required for Catabolism of Agropine by *Agrobacterium* Strains

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Received 10 March 1997/Accepted 30 May 1997

The mechanisms that ensure that Ti plasmid T-DNA genes encoding proteins involved in the biosynthesis of opines in crown gall tumors are always matched by Ti plasmid genes conferring the ability to catabolize that set of opines on the inducing *Agrobacterium* strains are unknown. The pathway for the biosynthesis of the opine agropine is thought to require an enzyme, mannopine cyclase, coded for by the *ags* gene located in the T_R region of octopine-type Ti plasmids. Extracts prepared from agropine-type tumors contained an activity that cyclized mannopine to agropine. Tumor cells containing a T region in which *ags* was mutated lacked this activity and did not contain agropine. Expression of *ags* from the *lac* promoter conferred mannopine-lactonizing activity on *Escherichia coli*. *Agrobacterium tumefaciens* strains harboring an octopine-type Ti plasmid exhibit a similar activity which is not coded for by *ags*. Analysis of the DNA sequence of the gene encoding this activity, called *agcA*, showed it to be about 60% identical to T-DNA *ags* genes. Relatedness decreased abruptly in the 5′ and 3′ untranslated regions of the genes. *ags* is preceded by a promoter that functions only in the plant. Expression analysis showed that *agcA* also is preceded by its own promoter, which is active in the bacterium. Translation of *agcA* yielded a protein of about 45 kDa, consistent with the size predicted from the DNA sequence. Antibodies raised against the *agcA* product cross-reacted with the anabolic enzyme. These results indicate that the agropine system arose by a duplication of a progenitor gene, one copy of which became associated with the T-DNA and the other copy of which remained associated with the bacterium.

Members of the genus *Agrobacterium* have evolved a unique interaction with certain plant species. Virulent agrobacteria harbor large extrachromosomal elements called Ti and Ri plasmids, a segment of which, the T region, is transferred from the bacterium to the susceptible plant during infections (6). Following transfer, this region, which then is called T-DNA, becomes integrated into the nuclear genome of the plant cell. Expression of hormone biosynthesis genes (*onc*) present on the integrated T-DNA results in the transformation of the normal plant cell into a crown gall tumor cell. The tumors characteristically produce novel low-molecular-weight metabolites called opines, whose synthesis also is directed by genes present on the T-DNA. Remarkably, although the T-DNA *onc* and opine biosynthesis (*ops*) genes are present on a bacterial replicon, they are expressed properly only in plant cells. Both gene classes lack identifiable bacterial promoters but contain 5′ and 3′ sequence motifs characteristic of plant transcriptional signals. In turn, the causative bacteria can utilize the opines produced by the tumors as a source of carbon, nitrogen, and energy (25, 28). Thus, *Agrobacterium tumefaciens* redirects plant cell metabolism to produce specific metabolites which the bacterium can use as growth substrates.

This correlation between opine production by the transformed plant tissue and opine catabolism by the causative bacterium led to the concept that these novel metabolites are the driving force mediating the interaction between *Agrobacterium* and its host plants (35). In this model, the opines provide a selective advantage to the inducing agrobacteria that are able to utilize these compounds as nutritional resources. This hypothesis, called the opine concept, is supported by recent work demonstrating that bacteria able to catabolize opines have an advantage over noncatabolizers when colonizing opine-producing plants (11, 12, 34, 38).

The opine concept is important for two reasons. First, in its general form it predicts that successful interactions between microbes and their eukaryotic hosts can be based, at least in part, on the capacity of the microorganism to specifically utilize a nutritional resource produced by the host. At a more fundamental level, the development of such an interaction predicts the coevolution of genes, within both the host and the microbe, that are responsible for the physiological relationship between the two. The opine systems associated with the Ti and Ri plasmids of *Agrobacterium* species represent a unique and intriguing example of such coevolution. These opine systems are binary in nature: the *ops* genes located in the T region function in the plant and are driven by plant-active promoter sequences, while the genes coding for the proteins involved in catabolism by the bacterium are driven by prokaryotic promoters.

The mechanisms by which the genes specifying these paired systems evolved or were acquired by the Ti plasmids remain unknown. In the cases of the octopine and the nopaline systems, the genes coding for the primary catabolic enzymes, as

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well as the proteins themselves, are unrelated to those of the corresponding T regions that specify the biosynthesis functions (39). Thus, for these two systems, the *ops* genes that specify opine biosynthesis in the plant host apparently arose independently of the genes that confer utilization of the corresponding opine by the bacterium. However, other mechanisms also may be operative. The T regions of some Ti and Ri plasmids code for a family of four mannose-containing opines called mannopinic acid, mannopine, agropinic acid, and agropine (8). As expected, the virulence plasmids carrying these T regions confer on the bacteria the capacity to utilize these four opines. Two of the genes required for catabolism of mannopine by the bacteria are related to two of the T-region genes responsible for production of mannopine by the transformed plant cells (20). In addition, we have identified an enzymatic activity, called catabolic mannopine cyclase, that in the bacterium catalyzes the lactonization of mannopine to agropine (7). This reaction is identical to that proposed to be responsible for the biosynthesis of agropine from mannopine in crown gall tumors (10, 22, 29). The similarity between these two lactonizing activities, one associated with opine synthesis in the plant tumor and the other associated with opine catabolism by the bacterium, suggested to us that the genes specifying these functions might have arisen from a common ancestor. This apparently is the case. In this report, we show that the T-region gene responsible for the biosynthesis of agropine by transformed plant cells and a gene located on another portion of the same plasmid that is required for the catabolism of this opine by the inducing bacterium not only code for enzymes with similar primary activities but also share a recent common ancestry.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The octopine-type strain Ach5 and its mutant derivative, strain 2408, harboring a Tn*5* insertion in *ags* (29) were used to produce crown gall tumors. *Escherichia coli* LCD (*met thi recA* Phr^+; a gift from L. C. DeVeaux) was used as the host for maxicell analysis. *E. coli* DH1 and DH5α (30) were used for cloning DNA fragments. Plasmid pYDH208 is a cosmid clone containing an insert of DNA from pTi15955 that encodes enzymes involved in the catabolism of mannopine and agropine as well as mannopine cyclase (9). Plasmid pYDPH208 is a derivative of pYDH208 that contains a nonpolar deletion mutation in *agcA* (18). The plasmid confers the ability to grow on mannopine but not on agropine. The other bacteria and plasmids and the culture conditions and media have been described previously (17–19).

Materials and reagents. Mannopine and agropine were synthesized according to previously published procedures (7, 28).

DNA manipulations. Plasmid DNA was isolated from *E. coli* and from *Agrobacterium* strains as described previously (17). Restriction enzymes, purchased from BRL (Gaithersburg, Md.) or New England BioLabs (Beverly, Mass.), were used according to the manufacturers' instructions. T4 DNA ligase and *Bal*31 nuclease were purchased from BRL. Plasmid DNA was introduced into *E. coli* and *Agrobacterium* hosts by transformation (15, 30).

DNA sequencing and computer analysis. The complete double-stranded nucleotide sequence was determined by the dideoxy chain termination method (32) with the Sequenase kit, version 2 (U.S. Biochemical Corporation), and [α-^{35}S] dATP (>1,000 Ci/mmol; Amersham, Inc.). The sequence was assembled and analyzed with the DNA Star software package (DNASTAR, Inc., Madison, Wis.). Databases were searched for related nucleotide and amino acid sequences with the FASTA (27) and BLAST (1) programs. Multiple alignments were produced with the Clustal V program (14).

Construction of a clone expressing anabolic mannopine cyclase. Plasmid pMAS4, which contains the mannityl opine biosynthesis genes from the T_R region of pTi15955 (33), was used as the template to amplify the *ags* gene by PCR. As primers, two oligonucleotide sequences (5′-CCAAGCTTG**ATG**GAC CTGTCCAAGC-3′ and 5′-CCGAATTCCATACTAATAAATGCGT-3′) were synthesized to generate a copy of the *ags* coding region clonable as an in-frame fusion to the *lacZ′* sequence. The bold triplet indicates the start codon of *ags* (3; corrected in reference 4), and the underlines represent the *Hin*dIII (first sequence) and *Eco*RI (second sequence) linker sites. The amplified fragment was cloned in pTZ19 (24) and recovered by transformation into *E. coli*. One such clone, called pAMC1, was shown by DNA sequence analysis to contain *lacZ′* fused in frame to the 5′ end of *ags*.

In vitro protein analysis. Proteins encoded by recombinant clones were expressed and labeled with [^{35}S]methionine (Tran^{35}S-Label, 1,078 Ci/mmol; ICN Biochemicals) by using a coupled transcription-translation system (Amersham, Inc.) or by the maxicell procedure (31). Proteins were separated by electrophoresis on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels as described by Laemmli (23). The gels were dried under vacuum, and radiolabeled proteins were detected by exposing the gels to Kodak X-OMAT film.

Production of axenic crown gall tumor lines. Ten-week-old plantlets of *Nicotiana tabacum* cv. Xanthi (XHF D8), germinated aseptically from surface-sterilized seeds, were wounded at the apex and inoculated with *A. tumefaciens* strains. Six weeks after inoculation, tumors were excised from the plants and propagated on half-strength MS medium (26) supplemented with 20 g of sucrose per liter and 500 μg of cefotaxime per ml. After 1 to 2 weeks on this medium, cultures of tumors were propagated on the same medium without cefotaxime. Axenic lines were identified by plating crude extracts from macerated tissues on Luria-Bertani agar. The presence of opines in these lines was assessed by high-voltage paper electrophoresis at pH 1.9 (28).

Preparation of extracts from normal and tumor callus tissues. About 6 g of axenic normal or tumor tissue was placed in a mortar and frozen with liquid nitrogen. Two hundred milligrams of glass beads (60-μm diameter) were added to the frozen tissue, and the mixture was ground to a fine powder. All further steps were performed at 4°C. The powder was mixed (1:1 [wt/vol]) with extraction buffer (50 mM potassium phosphate [pH 7.5], 0.1 mM EDTA, 1 mM β-mercaptoethanol, and 10% [wt/vol] Polyclar AT [Serva Biochemicals]), and the mixture was gently stirred for 30 min and then centrifuged at 10,000 × *g* for 15 min. The supernatant was recentrifuged at 20,000 × *g* for 15 min to yield about 7 ml of clear plant cell extract. Low-molecular-weight compounds, including endogenous opines, were removed by filtration over a column (30-ml bed volume) of Sephadex G25 (Pharmacia) equilibrated with extraction buffer. Fractions containing proteins were pooled and concentrated 10-fold under a stream of nitrogen. The concentrated extracts were used as the source of enzyme for anabolic mannopine cyclase assays.

Assay for mannopine cyclase activity. Mannopine cyclase activity was assayed as previously described (7). Precursor and product were separated by electrophoresis on Whatman 3MM paper and visualized with an alkaline silver nitrate reagent as described previously (28, 36).

Determination of the N-terminal sequence. Approximately 1 nmol of purified catabolic mannopine cyclase, purified as described by Hong and Farrand (19), was dialyzed against 0.01% trifluoroacetic acid in water and subjected to automated Edman degradation with an Applied Biosystems Model 477A sequencer located in the University of Illinois Biotechnology Center.

Preparation of polyclonal antiserum and immunoblot analysis. Murine polyclonal antiserum directed against purified catabolic mannopine cyclase was obtained from the Hybridoma Laboratory facility at the University of Illinois. Cells of DH5α(pAMC1), DH5α(pTZ19), and DH5α(pUC18PSS) were grown overnight at 37°C in L broth. Cell extracts were prepared by lysozyme treatment, sonication, and centrifugation. Proteins in the extracts were separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes as described by the manufacturer (Bio-Rad, Richmond, Calif.). The blocked membrane was incubated with the antiserum (1/1,000 dilution) and then with goat anti-mouse immunoglobulin G conjugated with alkaline phosphatase (Sigma Chemical Corp., St. Louis, Mo.). Reactions were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Nucleotide sequence accession number. The nucleotide sequence of *agcA* from pTi15955 has been deposited in the EMBL and GenBank libraries under accession no. Z23166.

RESULTS

Anabolic mannopine cyclase activity in crown gall tumors. Previous genetic studies (10, 22, 29) indicated that agropine is synthesized from mannopine by the product of the *ags* gene (also called *mas0*) located in the *mas* cluster of the T_R region of octopine-type Ti plasmids. However, there is no enzymatic data supporting this hypothesis. We identified this activity by assaying protein extracts prepared from axenic tumor tissues for their ability to cyclize mannopine to agropine. The endogenous opines present in these extracts were removed by column chromatography, and the mannopine lactonizing activity was assayed in vitro. Extracts from tumors induced by the wild-type *A. tumefaciens* Ach5 contained significant levels of lactonizing activity (Fig. 1). Extracts from normal tobacco callus tissue and from tumors induced by strain 2408, which contains an insertion mutation in *ags*, did not contain detectable levels of this activity. Enzymatic activity was correlated with the presence of agropine in untreated extracts from tumors produced by strain Ach5 (data not shown). Tumors induced by strain 2408 contained mannopine, mannopinic acid, and agropinic acid but did not contain any detectable agropine. Extracts from normal

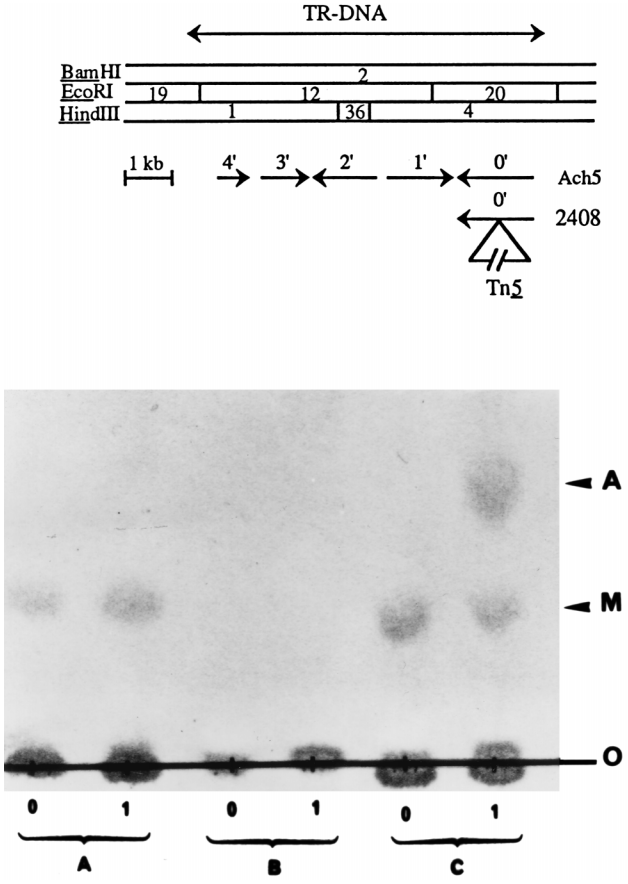


FIG. 1. The T-region *ags* gene codes for an activity that cyclizes mannopine to agropine in crown gall tumors. (Top panel) The physical and functional organization of the T_R region of octopine-mannityl opine-type Ti plasmids, such as pTiAch5, pTiB6, and pTi15955. The transcripts corresponding to the five ORFs identified in the T_R DNA and their transcriptional orientations are represented by arrows. Transcript 0′ corresponds to the *ags* gene. Strain 2408 harbors a Ti plasmid with a T_R identical to that of Ach5, its wild-type parent, except for a Tn*5* insertion located in the *ags* gene. (Bottom panel) High-voltage paper electrophoretic analysis of mannopine cyclase activity. Extracts prepared from tumors and from normal tobacco tissue were incubated with (lane sets A and C) or without (lane set B) mannopine as described in Materials and Methods. Samples taken at zero time (0) and at 1 h after the start of the reaction (1) were subjected to paper electrophoresis in formic-acetic acid (pH 1.9), and the substrate and product were visualized with alkaline silver nitrate, all as described in Materials and Methods. Lanes A, extract from a tumor induced by strain 2408 (a similar result was obtained when mannopine was incubated with extraction buffer only); lanes B, extract from a tumor induced by strain Ach5 (mannopine was not included in the reaction mixture for the enzyme assay) (a similar result was obtained with an extract from a tumor induced by strain 2408); lanes C, extract from a tumor induced by strain Ach5 (mannopine was included in the reaction mixture for the enzyme assay). Positions at which authentic agropine (A) and mannopine (M) migrate are indicated by arrowheads at the left margin of the electrophoretogram. O, origin of electrophoresis.

tobacco tissues did not contain detectable amounts of any of the four mannityl opines (data not shown).

***ags* codes for a mannopine cyclase activity when expressed in *E. coli*.** Given the lactonizing activity seen in the axenic tumors, the anabolic and catabolic enzymes appear to catalyze the same reaction. We tested this directly by assaying mannopine cyclase activity in extracts of bacteria harboring *ags*. A recombinant clone, pMAS4 (33), containing the *ags* gene with its native plant-active promoter region did not express detectable levels of lactonizing activity in *E. coli* or in a Ti plasmid-cured *A. tumefaciens* strain grown in minimal or rich medium (data not shown). However, extracts of *E. coli* harboring pAMC1, which contains *ags* translationally fused to *lacZ′*, contained high levels of an activity that cyclized mannopine to agropine (Fig. 2). Extracts prepared from *E. coli* harboring the vector pTZ19 did not contain such an activity.

Localization of the catabolic mannopine cyclase gene. Bacterial cells harboring pYDH208 express catabolic mannopine cyclase activity (9, 17). This clone, which contains a 21-kb insert mapping to the 4-o'clock region of the octopine-type Ti plasmid pTi15955 (9), confers all of the transport and enzymatic activities required for catabolism of mannopine and agropine (9, 17, 18, 20, 21) (Fig. 3). We localized the gene coding for catabolic mannopine cyclase activity by subcloning a series of fragments from pYDH208 (Fig. 3). Based on the analysis of mannopine cyclase activities conferred by pUC19KSS and pUC19KSP, we determined that the rightward end of the mannopine cyclase determinant, called *agcA*, is located between the *Sal*I and *Sph*I sites of the insert. The leftward end of *agcA* is located between the left *Eco*RI and *Hin*cII sites, as defined by plasmids pUC18BE1.9 and pUCBX#9. These results are consistent with the observation that subclone pUC18PSS, which contains a 1.6-kb *Pvu*I-*Sph*I fragment in pUC18, confers mannopine cyclase activity on *E. coli* (Fig. 2 and 3).

DNA sequence analysis. The complete double-stranded sequence of the 1.6-kb region that confers catabolic mannopine cyclase activity on host bacteria was determined (Fig. 4). Computer analysis revealed a single significant open reading frame (ORF). The putative *agcA* ORF is 1,206 bp long and could code for a protein of 402 amino acids with a predicted molecular weight of 43,793. This size is in good agreement with that of the protein produced by in vitro translation of subclones conferring mannopine cyclase activity (see below). This size also agrees with that calculated from size exclusion chromatography of the purified mannopine cyclase protein (19).

Relatedness of the genes coding for the catabolic and anabolic mannopine cyclase activities. The nucleotide sequence of *agcA* shows strong homology with the nucleotide sequences of the anabolic mannopine cyclase genes contained in the T_R regions of pTi15955 (Ti-*ags*) and pRiA4 (Ri-*ags*) (Fig. 4). When aligned, the coding regions of the three genes are 52% identical. In paired alignments *agcA* and Ti-*ags* are 61% identical, while *agcA* and Ri-*ags* show 65% identity (Table 1). In

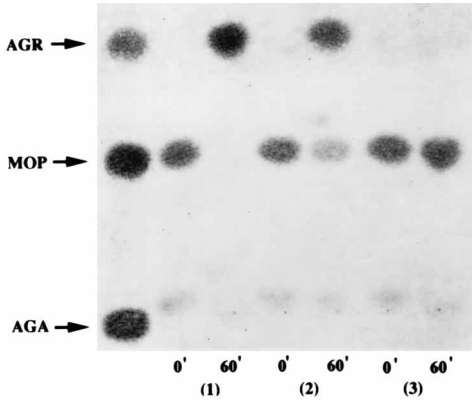


FIG. 2. *ags*, expressed in *E. coli*, confers mannopine cyclase activity. Cell extracts prepared from *E. coli* strains were incubated with mannopine as described previously (17). Samples, taken at zero time (0′) and at 60 min (60′) following initiation of the reaction were analyzed by paper electrophoresis as described in the legend to Fig. 1. Lanes 1 to 3 contain extracts from bacteria harboring pUC18PSS (*agcA*), pAMC1 (*ags*), and pTZ19, respectively. Abbreviations: AGR, agropine; MOP, mannopine; AGA, agropinic acid.

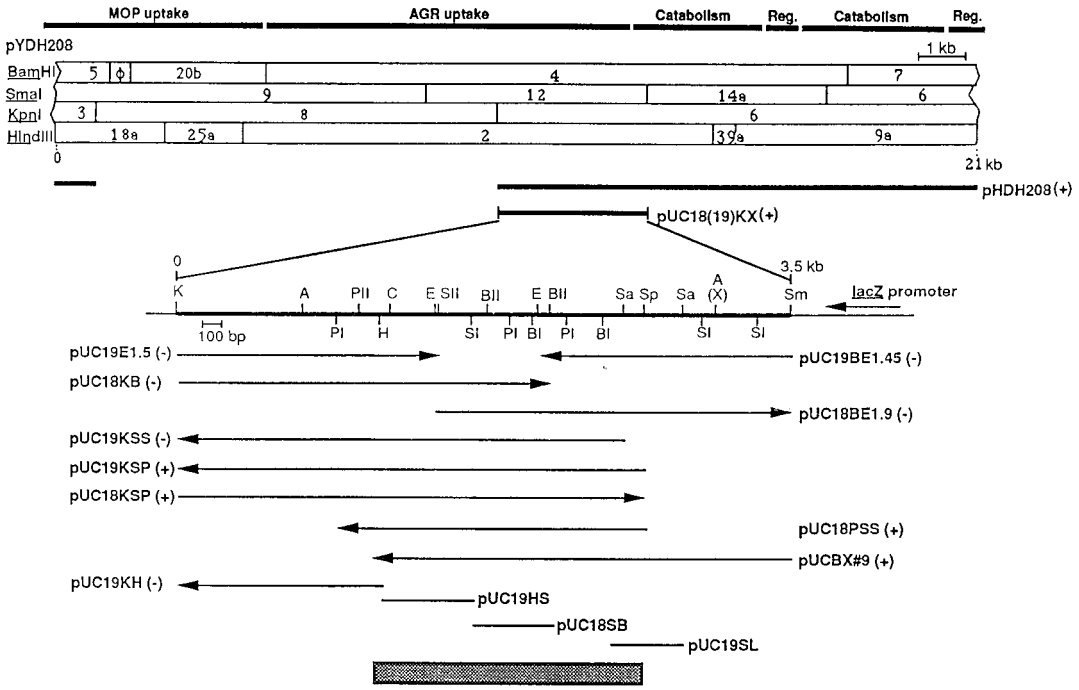


FIG. 3. Subcloning and localization of *agcA*, the gene coding for catabolic mannopine cyclase. The restriction map of pYDH208, which contains a 21-kb insert from the *moc* region of pTi15955, is shown at the top. This plasmid confers growth on mannopine (MOP) and agropine (AGR) and also encodes mannopine cyclase activity (9). Functional domains, determined as described in references 16 to 18, 20, and 21, are shown above the restriction map. pUC18(19)KX is pUC18 (or pUC19) containing a 3.5-kb insert derived from pYDH208, which encodes mannopine cyclase activity in *E. coli*. Arrows below the map of pUC18(19)KX indicate subclones in pUC18 or pUC19 derived from the plasmids pUC18(19)KX and pYDH208. Arrowheads indicate the direction of transcription with respect to the *lac* promoter of the vector. (+) and (−) indicate the presence and absence, respectively, of mannopine cyclase activity associated with the clone. The filled box at the bottom represents the region containing the catabolic mannopine cyclase gene. K, *Kpn*I; A, *Ava*I; PI, *Pvu*I; PII, *Pvu*II; H, *Hin*cII; C, *Cla*I; E, *Eco*RI; SI, *Sst*I; SII, *Sst*II; BI, *Bgl*I; BII, *Bgl*II; Sa, *Sal*I; Sp, *Sph*I; Sm, *Sma*I; (X), *Xho*I within the *Ava*I site; Reg., regulatory region.

comparison, the two anabolic mannopine cyclase genes are 68% identical (4) (Table 1). The 3′ noncoding regions of all three genes are distinguished from the coding regions by a sudden lack of significant sequence homology (Fig. 4). However, at the 5′ ends, the noncoding regions retain higher levels of identity; there is 27% identity between *agcA* and Ti-*ags*, 35% identify between *agcA* and Ri-*ags*, and 46% identity between Ti-*ags* and Ri-*ags* in the regions (coordinates 50 to 200) upstream of the three genes (Table 1).

The high degree of nucleotide sequence identity within the coding regions of the three genes is reflected at the derived amino acid sequence levels. The relatedness values between the three mannopine cyclase proteins range from 60 to 70% identity and from 80 to 85% similarity when conservative amino acid substitutions are included (Fig. 5).

While each of the three proteins is related to the other two throughout its entire sequence, homology plot analysis indicated that conservation is strongest in the C-terminal half (Table 1). The lowest level of relatedness between AgcA and the two Ags proteins occurs within the region lying between amino acid residues 112 and 194 (Fig. 5). This region is strongly conserved in the two anabolic mannopine cyclase proteins. Within this region the Ti and Ri plasmid *ags* gene products contain four and five more cysteine residues, respectively, than does AgcA (Fig. 5). Three of these cysteine residues are at conserved positions in the two anabolic enzymes.

***agcA* codes for a protein of the predicted size.** Several subclones carrying *agcA* were examined for the expression of proteins in a coupled in vitro transcription-translation system. All clones that yielded mannopine cyclase activity resulted in the production of a 45-kDa protein (Fig. 6). Indistinguishable results were obtained when these clones were examined in *E. coli* maxicells (data not shown). Subclones pUC19KX and pUC19KSP yielded an additional band, representing a protein of approximately 40-kDa, which is coded for by a gene directly downstream of *agcA* (16).

N-terminal sequence of AgcA. The nucleotide sequence of *agcA* presents two potential ATG start codons, each preceded by a reasonable consensus ribosomal binding site (Fig. 4). The sequence of the first 10 amino acid residues of purified catabolic mannopine cyclase protein, determined as described in Materials and Methods, matched that predicted by the nucleotide sequence, given the initiation of translation at the first ATG codon (position 205) (data not shown).

FIG. 4. Nucleotide sequence of *agcA* and its alignment with the nucleotide sequences of the *ags* genes from the T regions of a Ti and an Ri plasmid. The sequence of the *agcA* gene, determined as described in Materials and Methods and Results, was aligned with the sequences of the *ags* genes from pTi15955 (Ti-*ags*) and pRiA4 (Ri-*ags*) with the Clustal V program. The sequence for the Ti plasmid gene was from Barker et al. (3) as corrected by Bouchez and Tourneur (4). The sequence for the Ri-*ags* gene was from Bouchez and Tourneur (4). Gaps (−) were introduced to optimize the alignments. Vertical bars indicate nucleotide identities between any two of the sequences. Single dots represent identities between Ti-*ags* and Ri-*ags*. Asterisks denote nucleotides that are identical among all three of the sequences. The ATG and TGA triplets shown in boldface type denote the probable translational initiation and termination codons of the three genes. The underlined nucleotides in the Ti-*agcA* sequence represent a possible bacterial ribosomal binding site. The overlined nucleotides denote the TATA box motifs of the Ri and Ti *ags* genes.

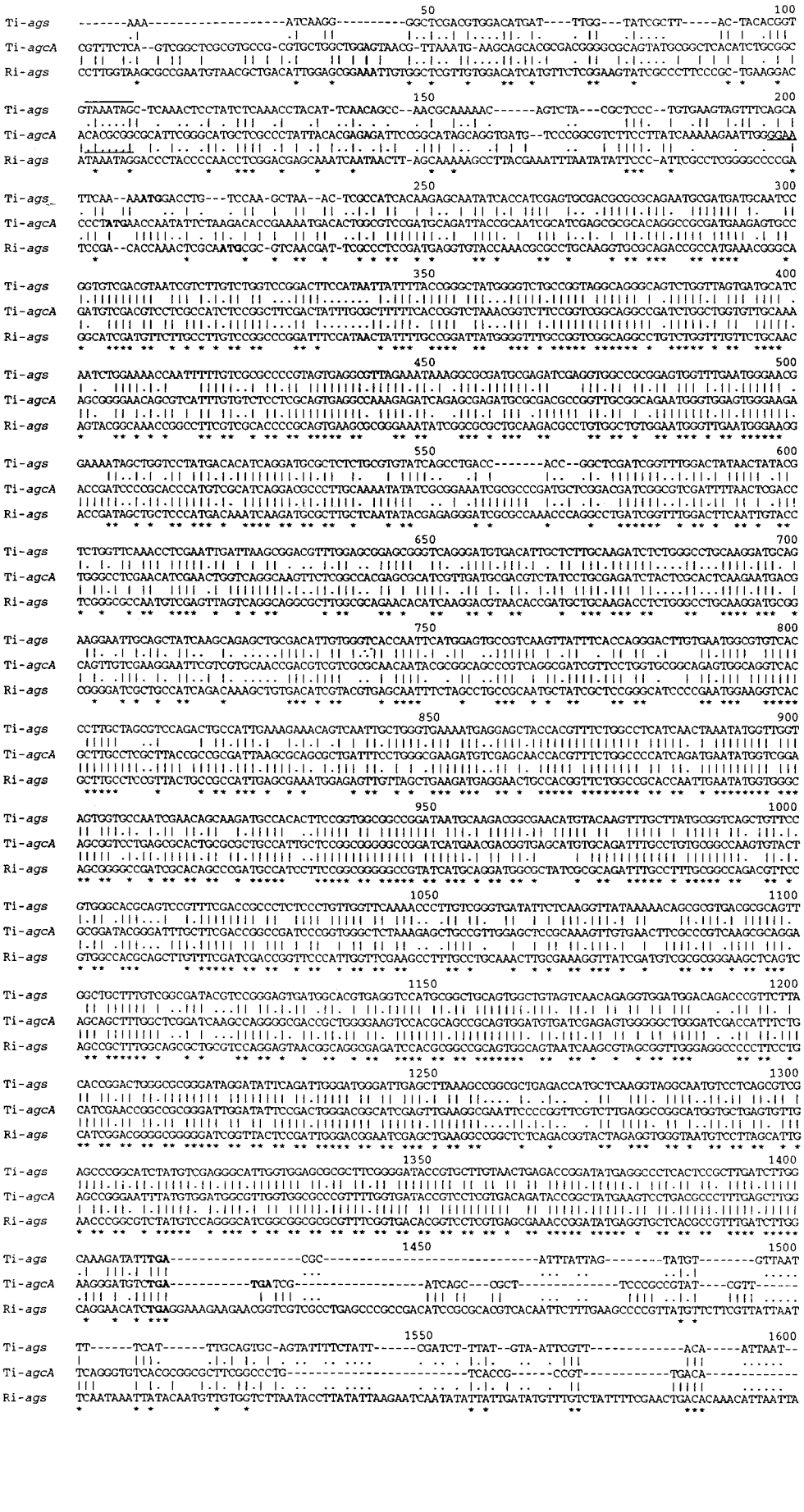


TABLE 1. Relatedness among genes for mannopine cyclase and their encoded products

Genes	Nucleotide sequence identity (%)			Amino acid sequence relatedness (%)			
	5′ Noncoding region	Coding region	3′ Noncoding region	Overall identity	Overall similarity	N-region identity[a]	C-region identity[b]
agcA and Ti-*ags*	26.5	60.9	6.3	58.0	79.0	45.9	70.8
agcA and Ri-*ags*	35.5	65.1	15.3	64.1	83.5	53.6	72.5
Ti-*ags* and Ri-*ags*	45.8	69.4	15.3	68.8	85.5	63.2	73.6
agcA, Ti-*ags*, and Ri-*ags*	18.0	51.5	4.8	51.5	74.5	40.5	64.5

[a] The N region extends from amino acid residues 1 to 219 of AgcA.
[b] The C region extends from amino acid residues 220 to 402 of AgcA.

***agcA* is expressed from its own regulated promoter.** Previous analysis suggested that *agcA* can be expressed from a promoter directly upstream of the ATG start codon (18). To confirm this, we constructed three plasmids in which *agcA* is preceded by increasing amounts of upstream sequence (Fig. 7). When assayed in strain NT1, which lacks regulatory genes and a mannopine transport system, plasmids pKS129ΔSm and pKS129ΔH both conferred high levels of mannopine cyclase activity (Fig. 7). This activity did not require pregrowth with the opine. These two plasmids also expressed mannopine cyclase activity when tested in NT1 harboring pYDPH208, a cosmid clone that contains the genes for a mannopine transport system and two genes specifying putative transcriptional repressors (20) but contains a nonpolar deletion mutation in *agcA* (18). However, the levels of lactonizing activity detectable from pKS129ΔSm and pKS129ΔH were substantially lower in this strain compared to the levels observed in a derivative strain of NT1 lacking pYDPH208 (Fig. 7). Preincubation of these two strains with mannopine resulted in detectably higher levels of activity compared to the levels detected in uninduced strains (Fig. 7). pKS129ΔSp, in which the 5′ deletion extends to within 25 bp of the translational initiation codon, did not confer detectable levels of the enzyme activity on strain NT1 or on strain NT1 harboring pYDPH208 (Fig. 7). These results are consistent

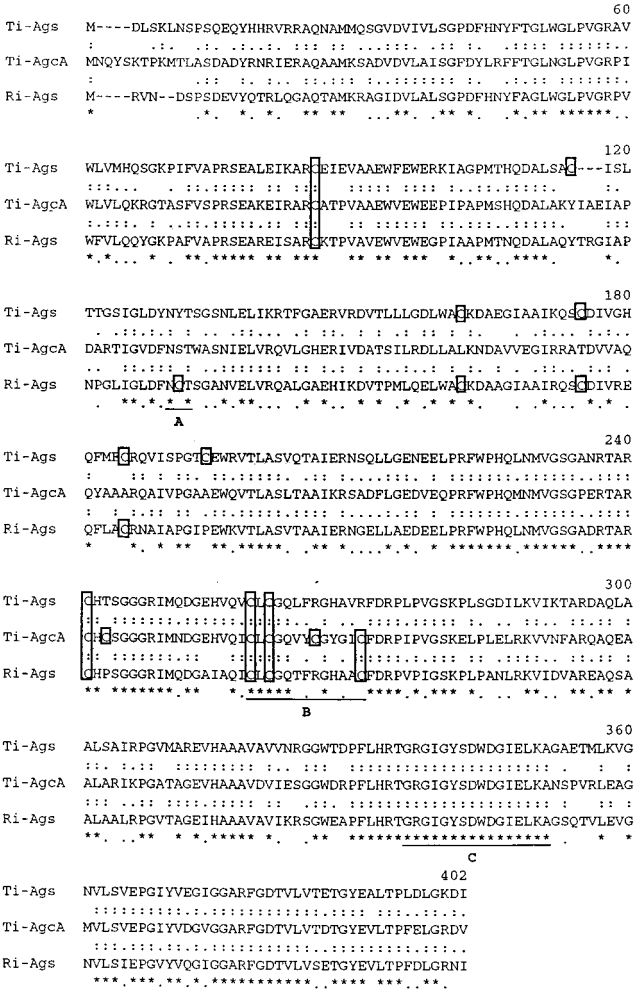


FIG. 5. Amino acid sequence relatedness between mannopine cyclase proteins. The derived amino acid sequence for *agcA* (Ti-AgcA) was aligned with the derived amino acid sequences for the *ags* genes from pTi15955 (Ti-Ags) and pRiA4 (Ri-Ags) with the Clustal V program. Colons and dots between amino acids represent identical and conservative residues, respectively. Asterisks and dots below the sequence indicate identities and conservative substitutions, respectively, common to all three proteins. Cysteine residues are boxed.

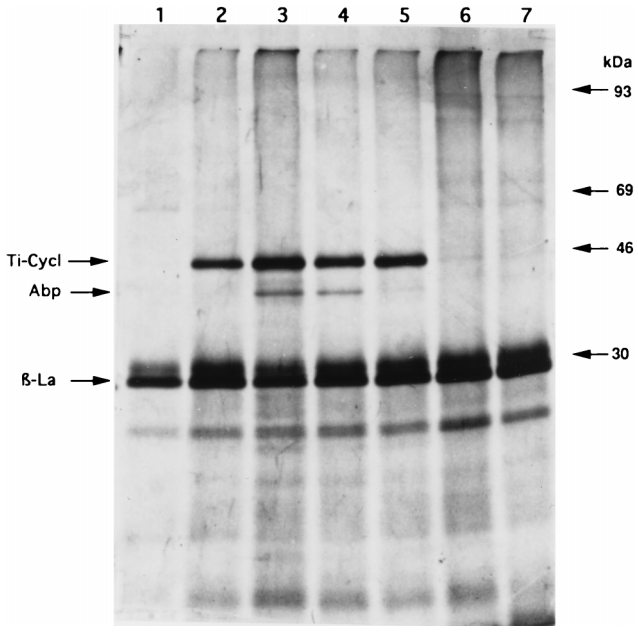
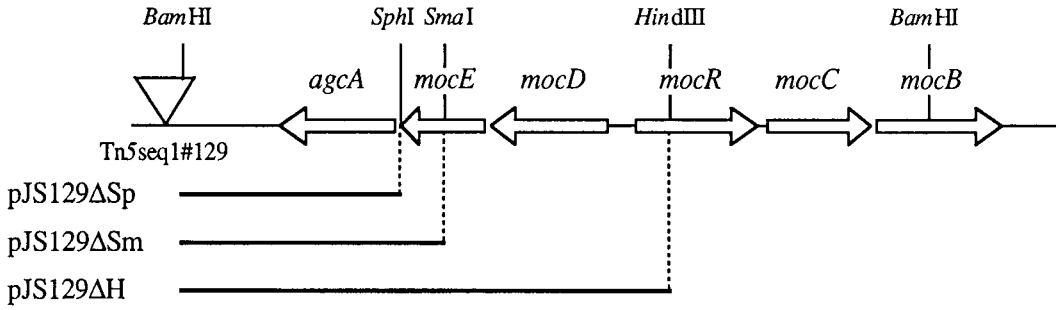


FIG. 6. Expression of products by subclones of pYDH208 conferring mannopine cyclase activity. Subclones derived from pYDH208 as described in the legend to Fig. 1 were expressed in an in vitro coupled transcription-translation system, as described in Materials and Methods. Translation products, labeled with [^{35}S]methionine, were separated by electrophoresis in polyacrylamide gels and detected by autoradiography. Lanes contain translation products from plasmids as follows: lane 1, pUC19; lane 2, pUC18PSS; lane 3, pUC19KX; lane 4, pUC19KSP; lane 5, pUCBX#9; lane 6, pUC19KH; and lane 7, pUC19E1.5. Positions and sizes of marker proteins are shown on the right. Bands representing the β-lactamase (β-La) encoded by the vector and the mannopine cyclase protein (Ti-Cycl) as well as a protein of unknown function (Abp) are indicated by arrows on the left side of the autoradiogram.



Strain	MOP cyclase activity	
	Uninduced	Induced
NT1(pTi15955)	+	+++
NT1(pYDH208)	++	+++
NT1(pKS129ΔSp)	-	NT
NT1(pKS129ΔSm)	++	NT
NT1(pKS129ΔH)	+++	NT
NT1(pYDPH208)	-	-
NT1(pYDPH208, pKS129ΔSp)	-	-
NT1(pYDPH208, pKS129ΔSm)	+	++
NT1(pYDPH208, pKS129ΔH)	++	+++

FIG. 7. *agcA* is expressed from its own promoter. The map at the top shows the organization of the *moc* region of pTi15955 (20) and the structures of the three clones containing various amounts of native sequence upstream of *agcA*. The constructs were produced from pSaB4#129, a clone containing the *moc* region with a Tn*5*seq1 insertion located downstream of *agcA* (20). The lower portion of the figure shows the levels of mannopine cyclase activity, determined as described in Materials and Methods, in derivatives of *A. tumefaciens* NT1 precultured in the absence or presence of mannopine. The NT1 strain with the first five plasmids shown lacks a system of transporting mannopine into the cells. Symbols: −, no detectable activity; +, barely detectable activity; ++, conversion of between 25 and 75% of the mannopine to agropine; +++, conversion of >75% of the mannopine to agropine. NT, not tested.

with our proposal that the expression of *agcA* is driven by its own promoter and that this expression is regulated by the products of one or both of the repressor-like genes located on pYDH208 (20).

Anabolic and catabolic mannopine cyclases are immunologically related. Polyclonal antiserum raised against purified catabolic mannopine cyclase reacted strongly with a protein of about 45 kDa present in an extract of *E. coli* expressing *agcA* from pUC18PSS (Fig. 8). The antiserum also reacted strongly with a protein of about 40 kDa present in an extract of *E. coli* expressing active mannopine cyclase from the *ags* gene in pAMC1 (Fig. 8). No reaction was detected with any proteins in extracts from an *E. coli* strain containing the vectors used for these constructs.

DISCUSSION

Our enzymological and genetic analyses clearly show that, in crown gall tumors induced by an octopine-mannityl opine-type *A. tumefaciens* strain, the *ags* gene located on T_R codes for an enzymatic activity that converts mannopine to agropine. This is consistent with the pathway for the biosynthesis of the lactone opine proposed by several groups (10, 22, 29).

Remarkably, this activity is indistinguishable from that present in agrobacteria harboring Ti or Ri plasmids that confer catabolism of agropine (7). Our results show that the catabolic mannopine cyclase activity expressed in *Agrobacterium* is encoded by a gene, which we name *agcA*, that is distinct from but a phylogenetic homolog of the *ags* determinant located on the T region of the same plasmid as well as of the *ags* gene from an agropine-type Ri plasmid. *agcA* is located about 35 kb clockwise from *ags* on pTi15955 and is within a cluster of genes that confer on the bacterium the ability to catabolize mannopine and agropine (17, 20). *agcA* and the two *ags* genes exhibit about 70% nucleotide sequence identity, and their products have extensive amino acid sequence relatedness. Moreover, AgcA and the Ti plasmid Ags maintain common structural features as indicated by immunological cross-reactivity. The two proteins also retain functional identity. When expressed in *E. coli*, both enzymes catalyze the lactonization of mannopine to agropine (Fig. 2), and the anabolic mannopine cyclase, when expressed in *Agrobacterium* from a bacterial promoter, fully complements a mutation in *agcA* that abolishes catabolic mannopine cyclase activity as well as growth on agropine (18). Clearly, *agcA* and the two *ags* genes derived from a common ancestor. Based on the degrees of overall similarity between pairs of the three proteins, catabolic mannopine cyclase encoded by the Ti plasmid is more closely related to the anabolic mannopine cyclase encoded by the Ri plasmid than it is to the anabolic enzyme encoded by the Ti plasmid from which it was cloned.

Despite overall strong conservation throughout the proteins, a segment corresponding to residues 112 to 194 shows considerable variation between AgcA and Ags. This segment con-

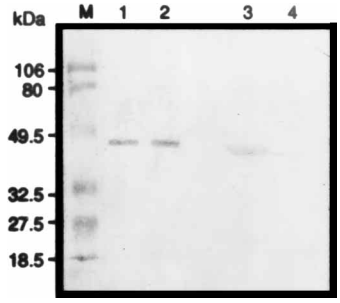


FIG. 8. Immunological cross-reactivity between catabolic and anabolic mannopine cyclase proteins. Extracts prepared from *E. coli* cells were separated by electrophoresis on an SDS-polyacrylamide gel, electrotransferred to a nitrocellulose membrane, and incubated with polyclonal antiserum raised against purified catabolic mannopine cyclase. Reacting proteins were visualized by an alkaline phosphatase system as described in Materials and Methods. Lanes: M, marker proteins with sizes indicated in kilodaltons; 1, 20 ng of purified catabolic mannopine cyclase; 2, total protein extract from DH5α(pUCP18PSS); 3, total protein extract from DH5α(pAMC1); 4, total protein extract from DH5α (pTZ19).

tains three cysteine residues (positions 162, 175, and 185) which are conserved only in the two plant-active Ags proteins (Fig. 5). Other cysteine residues either are conserved among two or all three of the proteins or are present in one but not the other two. This pattern of cysteine residues may reflect the evolutionary relationships among these three proteins since the codons for this amino acid have the smallest mutation probability. Alternatively, it may reflect requirements for activity in plant versus bacterial hosts. However, the pattern is not required for mannopine cyclase activity per se, since both AgcA and Ags exhibit enzymatic activity when expressed in *E. coli*. and *A. tumefaciens* (Fig. 2) (18).

Catabolism of agropine proceeds through mannopine and is dependent upon catabolic mannopine cyclase (18). We recently reported that the two Ti plasmid genes essential for catabolism of mannopine by the bacteria are related to *mas1* and *mas2*, the T-region genes responsible for synthesis of these opines by crown gall tumors (21). Moreover, mannopine oxidoreductase, coded for by one of these catabolic pathway genes, *mocC*, catalyzes the oxidation of mannopine to deoxyfructosyl glutamine (20). In the transformed plants, deoxyfructosyl glutamine is the direct precursor of mannopine, and the oxidation of mannopine to deoxyfructosyl glutamine by the catabolic enzyme is precisely the reverse of the reaction proposed to be associated with the biosynthesis of mannopine by Mas1, the homolog of mannopine oxidoreductase (10, 22, 29). Thus, the three genes required by the bacteria for catabolism of agropine via mannopine are related to the T-region genes responsible for biosynthesis of these opines by the transformed plant cells. Furthermore, as shown in Fig. 9, the pathway for catabolism in the bacterium most likely is the reverse of the pathway for biosynthesis in the plant. Interestingly, like the two mannopine cyclases, catabolic mannopine oxidoreductase catalyzes the reverse biosynthetic reaction, the reduction of deoxyfructosyl glutamine to mannopine, with high efficiency (21). That the two characterized catabolic enzymes, mannopine cyclase and mannopine oxidoreductase, catalyze the biosynthetic reactions with high efficiency does not, a priori, pose a problem. Presumably, substrate flux into pathways for general metabolism ensures that concentrations of the intermediates of opine catabolism are kept at levels low enough to maintain the pathway in the catabolic direction. However, the obvious biosynthetic capacity of the two enzymes is consistent with our hypothesis that the pathway, functioning in the anabolic direction, may constitute a novel strategy used by the bacterium for sequestering the mannityl opines in the face of competition by other microorganisms able to utilize these substrates (18, 21).

Our results show that the two gene pairs, *mas1-mocC* and *ags-agcA*, are phylogenetically related and code for enzymes with the same activities. This supports our hypothesis that gene duplication is responsible for the origin of the mannopine-agropine synthesis and catabolism system. However, mechanisms other than gene duplication also are responsible for the emergence of such opine systems. The anabolic and catabolic gene sets for the octopine and nopaline systems are unrelated and apparently arose independently of one another (39).

Although there is no doubt that *ags* and *agcA* have a common phylogeny, it is not clear whether the ancestral gene was of prokaryotic or eukaryotic origin. Codon usage analysis provides no clues; similar patterns of usage are exhibited by the three genes (16). Nor could we identify any significant bias when the codon usage patterns of the 3 mannopine cyclase genes were compared to those of a group of 13 plant genes, 8 genes of Ti plasmid T-DNA that are expressed in plants, and 20 *vir* genes also contained on the Ti plasmid but expressed in the bacterium (16). Analysis of the 5′ untranslated promoter regions of *ags* and *agcA* also failed to provide information concerning ancestry. The *ags* promoter is typically eukaryotic, containing TATA and CCAAT elements, upstream enhancer domains, and a negative control region (2). In the plant, this promoter produces relatively high levels of expression but does not yield detectable expression of *ags* in the bacterium. *agcA*, on the other hand, is expressed in the bacterium but is strongly regulated, being inducible to high levels only when mannopine or agropine are supplied in the medium (17). This substrate-

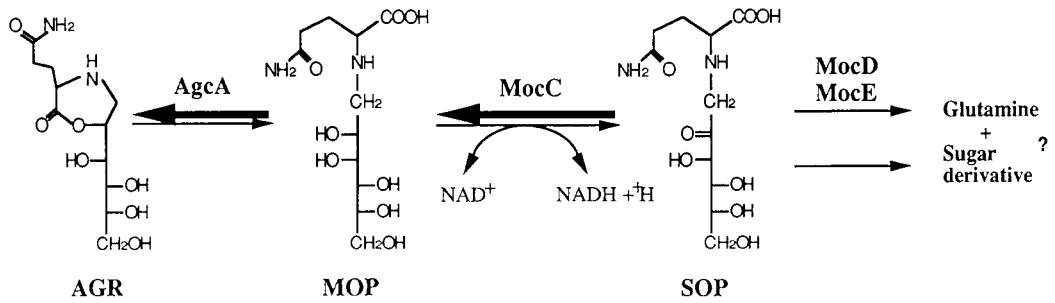


FIG. 9. Proposed pathway for the catabolism of agropine (AGR) and mannopine (MOP) specified by the *moc* region of octopine-mannityl opine-type Ti plasmids. AGR and MOP, produced by crown gall tumors, are taken up by cognate transport systems (16–18). AGR is converted to MOP by catabolic MOP cyclase (AgcA). MOP, either from AGR via MOP cyclase or from crown galls, is oxidized to deoxyfructosyl glutamine (santhopine [SOP]) by MOP oxidoreductase (MocC). The thick arrows indicate the favored reaction directions as determined in vitro (19, 21) and, for MOP cyclase, in vivo (7).

mediated induction is catabolite repressed by succinate, and this repression, in turn, is relieved in the presence of succinate when the opine is provided as the sole source of nitrogen (17). Thus, the prokaryotic promoter driving the expression of *agcA* is highly evolved and contains *cis*-acting elements that allow differential responses to changing environmental conditions that the bacterium might encounter. Our results show that *agcA* is expressed from its own promoter and that sequences immediately 5′ to the gene are sufficient for proper regulation (Fig. 7). While this promoter is unlike any other prokaryotic promoter sequence described to date, it shares features with promoter regions of other genes required for catabolism of the mannityl opines (20). Thus, both the anabolic and catabolic genes are expressed from their own promoters, and the two promoters are strongly adapted to the hosts in which they function. As might be expected, there is little homology between the nucleotide sequences of the promoter regions of these two genes (Fig. 4). This suggests that the control regions of the two genes have evolved rapidly to meet the requirements for regulated expression in their respective prokaryotic and eukaryotic hosts. Interestingly, there also is only moderate sequence identity between the promoter regions of the *ags* genes from the T regions of the Ti and Ri plasmids (4). This suggests either that the two genes arose in their T-region contexts independently or that the plant-active promoter regions have evolved divergently to accommodate the different plant tissue types in which they express *ags*. The fact that the overall organization of the three genes required for mannityl opine synthesis otherwise is identical in the T regions of the Ti and Ri plasmids (4) argues for the latter interpretation.

Searches of several DNA and protein databases failed to identify other genes or proteins related to *agcA*, *ags*, or their translational products. This suggests that the two mannopine cyclases comprise a novel class of lactonizing enzymes. However, Vaudequin-Dransart et al. (37) recently described a new family of opines present in crown gall tumors, including among its members deoxyfructosyl glutamine and chrysopine (5). These so-called chrysopine tumors do not contain mannopine or agropine. However, as described above, deoxyfructosyl glutamine is the immediate precursor of mannopine. More intriguingly, chrysopine is the lactone of deoxyfructosyl glutamine and is structurally related and conceptually analogous to agropine. Agrobacteria that degrade chrysopine contain an activity, called chrysopine cyclase, that cyclizes deoxyfructosyl glutamine to the lactone opine (5). Moreover, catabolic mannopine cyclase will lactonize deoxyfructosyl glutamine to a compound that is indistinguishable from chrysopine (19). However, the converse is not true; chrysopine cyclase does not recognize mannopine as the substrate (5). This suggests that mannopine cyclase and chrysopine cyclase also may have a common progenitor and strengthens the proposal that the chrysopine system may be the ancestor from which the mannityl opine system evolved (37).

We have proposed that catabolic mannopine cyclase evolved as a response by mannopine-type *Agrobacterium* strains to competition pressures placed on these bacteria by other soil microflora that have developed the ability to utilize mannopine (18). Indeed, the mannopine-type Ti and Ri plasmids may represent the progenitors of the agropine system. The T regions of these elements confer on tumors and hairy roots the ability to produce mannopine, mannopinic acid, and agropinic acid but not agropine. Moreover, these Ti and Ri plasmids, although conferring the ability to grow on the three mannityl opines produced by the neoplasias, do not allow the bacteria to utilize agropine. Nor do these bacteria express mannopine cyclase activity. The T region of one mannopine-type Ri plasmid contains close homologs of the *mas1* and *mas2* genes found on the T region of agropine-type Ri and Ti plasmids but lacks *ags* (13). Thus, the opine biosynthesis systems specified by the mannopine- and agropine-type T-DNAs differ only by the presence of *ags*. This suggests that the latter could have arisen from the former by duplication of *agcA* and its translocation, as *ags*, to the T region. If our hypothesis is true, it implies that the capacity of the bacteria to utilize agropine preceded the appearance of agropine-type crown gall tumors or hairy roots.

We speculate that the evolution of the agropine-based interaction between *Agrobacterium* and its plant host involved two steps. First, in the face of pressure from other soil microbes, the progenitor mannityl opine-type agrobacteria evolved *agcA* as a mechanism to sequester the mannopine, as agropine, in an intracellular form. This is consistent with our observation that exogenous mannopine is taken up and rapidly converted intracellularly to agropine by *A. tumefaciens* strains expressing mannopine cyclase (7). Then, as a function of the gene duplication event yielding *agcA-ags*, the *ags* gene was translocated to the T region. Upon *vir*-mediated transfer to plant cells, this relocated gene resulted in a change in the plant genotype and concomitant phenotype, such that the plant host then supplied to the bacterium a new opine, agropine, that is not utilizable by the competing microflora. Thus, a set of genetic alterations within the bacterium, resulting from selection pressures from outside, were transmitted to the plant. Here they effected a corresponding coevolution of the host to preserve the selective nature of the microbe-plant interaction conferred by the opine system.

ACKNOWLEDGMENTS

Portions of this work were supported by a grant from the Biotechnology Research and Development Corporation to S.K.F. and by funds from CNRS and INRA to Y.D.

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