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Analysis of *Trans*-Acting Elements for Regulation of *moc* Operons of pTi15955 in *Agrobacterium tumefaciens*

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Abstract Two putative regulator genes, *mocR* and *mocS*, of the *moc* (*m*annityl *o*pine *c*atabolism) operons in pTi15955 of the octopine-/mannityl opine-type *Agrobacterium tumefaciens* strain 15955, were tested for their possible roles as repressors in the *moc* operons. The regions upstream of *mocC* and *mocD*, the first structural genes in the two divergently oriented *moc* operons, were transcriptionally fused into the promoterless *lacZ* reporter gene. Each of the *lacZ*-fusions was introduced into *Agrobacterium* strain UIA5, a Ti plasmid-cured derivative, harboring either a *mocR* or a *mocS* clone. The resulting strains were grown in media containing various sugar sources, and the β-galactosidase activities were quantitatively measured. The results suggested that MocR repressed the expression of *mocC* and *mocD*. The expression of the fused β-galactosidase was not induced by mannopine (MOP) or possible catabolic intermediates of the opine, *e.g.* santhopine (SOP), glucose, mannose, or glutamine. However, the repression was significantly relieved by the supplementation of MOP and the concomitant introduction of the *agcA* gene encoding MOP cyclase that catalyzes the lactonization of MOP to agropine (AGR). These results suggested that AGR, rather than MOP or the other catabolic intermediates, is the inducer for the expression of the operon. On the contrary to previous report showing that the induction levels of *mocC* and *mocD* were lowered by the supplementation of inorganic nitrogen in media, the expression of these genes was not affected by the level of nitrogen in our reporter system. MocS did not strongly repress the expressions of *mocC* and *mocD*. It is possible that MocS may be involved in the regulation of the operons present downstream of the *moc* operon, which are responsible for the utilization of mannopinic acid and agropinic acid.

Key words: *Agrobacterium*, pTi15955, *moc*, *mocR*, *mocS*, promoter

Crown galls and hairy roots induced by *Agrobacterium* strains produce novel low molecular weight compounds called opines. The genes responsible for the synthesis of these opines in plant tumors are present in the T-region of Ti (tumor-inducing) or Ri (hairy root-inducing) plasmids of the pathogens [14]. The opines produced by the plant tumors can be utilized as carbon and energy sources by the disease-causing pathogens. Without exception, the Ti plasmid which confers the synthesis of an opine in infected plant cells, also carries genes for the utilization of that opine by the bacteria [2]. Mannityl opine utilization is a common property of various *Agrobacterium* strains [2, 13, 20]. Four opines belong to the mannityl opine family. Mannopine (MOP) and mannopinic acid (MOA) are imine conjugates of mannose and glutamine or glutamic acid, respectively. Agropine (AGR) is a cyclized derivative of MOP, and agropinic acid (AGA) is generated non-enzymatically from MOP and AGR by spontaneous rearrangement [19]. The pTi15955 plasmid from *Agrobacterium tumefaciens* strain 15955 is a classical octopine-type Ti plasmid, which confers the catabolism of the mannityl opines as well as octopine [3]. A cosmid clone, pYDH208 containing a 21-kb fragment of pTi15955, confers the transport and catabolism of MOP on Ti plasmid-cured agrobacterial strain, NT1, or on Ti plasmid- and pAtC58 cryptic plasmid-cured derivative, UIA5 [5]. At least four genes - *agcA*, *mocC*, *mocD*, and *mocE* - arranged as two divergently oriented operons, are essential for the catabolism of AGR (Fig. 1). AGR, transported by an AGR-specific ABC-type transport system, is decyclized to MOP by MOP cyclase encoded by *agcA* [6]. MOP, either converted from AGR by MOP cyclase or taken up by a MOP-specific ABC-type transport system [15], is oxidized by MOP oxidoreductase encoded by *mocC* [8]. A biochemical study of the MOP oxidoreductase showed that the enzyme converts MOP to deoxyfructosyl glutamine (dfg, or commonly called santhopine, SOP) at the expense of NAD^+ as a cofactor [8]. The product, SOP, is further degraded by the products encoded by *mocD* and *mocE* [9]. The final products are proposed to be glucose and glutamine, however, no direct biochemical evidence for these reactions has yet been shown. Nonetheless, genetic studies clearly

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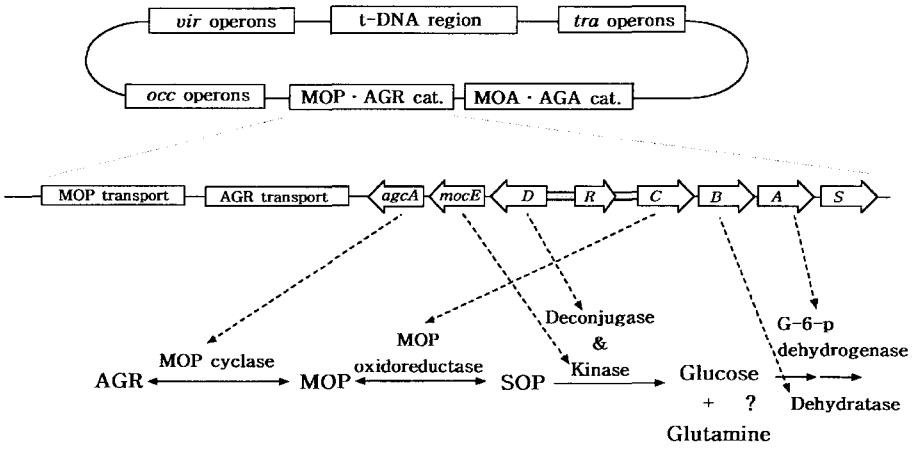

Fig. 1. Functional map of pTi15955 and proposed biochemical pathway for the degradation of AGR and MOP.
Functional map of pTi15955 and the structure of the *moc* operons; *vir*, virulence genes necessary for the tumorogenesis of the bacterium; *tra*, *tra* genes necessary for the conjugal transfer of the Ti plasmid; *occ*, octopine catabolism. (This figure is not drawn to scale.) AGR is reversibly converted to MOP by MOP cyclase encoded by *agcA*. The product is oxidized by MOP oxidoreductase encoded by *mocC*. The product SOP is further degraded into supposedly glucose and glutamine by enzymes encoded by *mocD* and *mocE*. The sugar product is further degraded through the Entner-Doudoroff pathway by a glucose-6-phosphate dehydrogenase and a dehydratase encoded by *mocA* and *mocB*, respectively.

indicate that the two genes are absolutely necessary and sufficient for the catabolism of SOP in a Ti-plasmid cured agrobacterial strain [8].

The regulation of the expression of the *moc* genes appears to be complicated. The Ti plasmid pTi15955 confers the utilization of four members of mannityl opines, and the genes for the functions are arranged as contiguous operons in a 45-kb region of the Ti plasmid [3]. Considering that the four members of the opines have close similarities in their chemical structures, it is possible that the expression of the genes for the utilization of each of the four opines may be systematically controlled. Moreover, the fact that agrobacterial cells can utilize the opines as the sole nitrogen source, makes it possible that an intracellular nitrogen level may affect the expression of the genes.

In a previous study by the current authors, a DNA sequencing analysis of the *moc* operons identified two putative repressors, *mocR* and *mocS* (Fig. 1). The deduced amino acid sequences of *mocR* and *mocS* showed high homology between each other. They also showed a relatedness to the repressors of *E. coli* operons for the utilization of various sugars including glucose, lactose, fructose, and galactose [9]. Genetic studies have shown that *A. tumefaciens* strain NT1, a Ti plasmid-cured derivative of strain C58, carrying pYDH208 with a mutation in either of these two genes, resulted in faster growth on a medium containing MOP as the sole carbon source than that of NT1 harboring a wild-type pYDH208 [5]. These findings suggested that *mocR* and *mocS* possibly encode negative regulators that modulate the expression of *moc* genes. However, no systematic study has been made to identify the roles of these genes in controlling *moc* genes.

In order to elucidate the roles of MocR and MocS in the regulation of the *moc* operon expression, the regions upstream of *mocC* and *mocD* genes (Fig. 1), which are thought to comprise *cis*-acting elements necessary for proper expression and regulation, were fused to promoterless reporter genes. The expression of the reporter gene was examined in the presence or absence of the putative negative regulators under various conditions. We report here that MocR, rather than MocS, significantly repressed the expression of the *moc* genes, and that the authentic inducer for the expression is AGR rather than MOP. We also tested the effect of inorganic nitrogen on the expression of *moc* genes, and showed that, on the contrary to previous report, nitrogen does not affect the induction level of *mocC* and *mocD* in our reporter system.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The strains and plasmids employed in this study are listed in Table 1.

Culture Media and Chemicals

Nutrient broth (NB, Difco) and L broth (LB, Difco) were used as rich media, and AT minimal medium was used as the minimal medium for the *Agrobacterium* strains [3]. *E. coli* cells were grown at 37°C, while *Agrobacterium* cells were grown at 28°C. For *E. coli* strains, spectinomycin (Sp) at 25 μg/ml, kanamycin (Km) at 25 μg/ml, tetracycline (Tc) at 10 μg/ml, and ampicillin (Ap) at 100 μg/ml were used. For *Agrobacterium* strains, spectinomycin, kanamycin, rifampicin (Rf), and carbenicillin (Cb) were used at 100

Table 1. Bacterial strains and plasmids employed in this study.

Strains or plasmids	Relevant property or genotypes	Sources or reference
Strains		
E. coli		
S17-1	RP4-2, Tc::Mu-Km::Tn7, *pro*, *rec*$^-$, *mod*$^+$, Tp^r	[18]
A. tumefaciens		
UIA5	pTiC58- and pAtC58-cured derivative of the C58	Our collection
Plasmids		
pRG970	Broad-host range promoter-probe vector, pVS1 origin, St^r/Sp^r	[21]
pDSK970	Broad-host range vector, a derivative of RSF1010, Km^r	[7]
pRK415	Broad-host range vector, IncP origin, Tc^r	[7]
pHP45	A derivative of pBR322 containing Ω fragment, St^r/Sp^r, Tc^s	[16]
pYDH208	A cosmid clone of pTi15955, MOP$^+$AGR$^+$, Tc^r	[3]
pYDH901	A cosmid clone of pTi15955, MOP transport$^+$, Tc^r	[3]
pBSE22a	*Eco*RI fragment 22a from pYDH208 cloned in pBluescript SK-	This study
pBSH9a	*Hin*dIII fragment 9a from pYDH208 cloned in pBluescript SK-	This study
pMC-*lacZ*	A derivative of pRG970, in which a 462-bp region upstream of *mocC* is fused to *lacZ*.	This study
pMD-*lacZ*	A derivative of pRG970, in which a 244-bp region upstream of *mocD* is fused to *lacZ*.	This study
pWH-R	A 1.2-kb *Sal*I-*Sph*I fragment containing *mocR* cloned in pDSK519	This study
pWH-S	A 1.5-kb *Pst*I fragment containing *mocS* cloned in pDSK519	This study
pWH-RS	A derivative of pDSK519 cloned with *mocR* and *mocS*	This study
pWH-4T	A 5.0-kb *Sma*I-*Xba*I fragment containing the MOP transport operon from pYDH901 cloned in pRK415	This study
pBSS12	A 5.0-kb *Sma*I fragment containing *agcA* from pYDH208 cloned in pBluescript SK-, Ap^r	This study
pWH-RAg	A concatamer between pWH-R and pBSS12, Cb^{r}Km^r	This study

Abbreviations; Tp, trimethoprime; St, streptomycin; Sp, spectinomycin; Km, kanamycin; Tc, tetracycline; Ap, ampicillin; Cb, carbenicillin.

μg/ml, and tetracycline was used at 1.5 μg/ml. Mannopine was purchased from Sigma Inc. (St. Louis, U.S.A.) and used at a final concentration of 5 mM.

The chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) (Sigma) and 5-bromo-4-choloro-3-indolyl-β-D-glucuronide (X-gluc) (Sigma) were used in a solid medium at a concentration of 40 μg/ml. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma) was used for liquid β-galactosidase assays.

DNA Manipulations

Large- and small-scale isolations of plasmid DNA were performed using a rapid alkaline lysis procedure [11]. Restriction enzyme digestions and ligation reactions were carried out as recommended by the manufacturers (Promega, Takara, and MBI).

PCR Conditions

The region upstream of *mocC* containing a promoter was PCR-amplified using primers C5 and C3 (Table 2) and the pBSE22a plasmid DNA as a template. The intergenic region between *mocD* and *mocR* was amplified using primers D and R (Table 2) and the pYDH208 plasmid DNA as a template. The reaction mixtures were prepared as recommended by the manufacturer (Promega), and the reactions were carried out according to the following program; step 1, 94°C for 30 sec; step 2, 52°C for 1 min; step 3, 72°C for 1 min; step 4, go on to step 1, repeat 37 times; step 5, 72°C for 10 min; step 6, 4°C. After PCR, the DNA nucleotide sequence of each product was determined to confirm the reactions.

MOP Uptake Test

Uptake of MOP was estimated indirectly by measuring the disappearance of the substrates from the liquid medium. The test strains were grown in 0.5 ml of ATNG liquid medium (AT minimal medium supplemented with 0.2% glucose and 0.15% ammonium sulfate) [3] for 30 h at 28°C with shaking. Cells were collected by centrifugation and washed with a 0.9% NaCl solution. The collected cells were resuspended in 100 μl of ATN minimal medium (AT medium containing 0.15% ammonium sulfate only) containing MOP at a final concentration of 1 mM as sole carbon source. The culture was incubated at 28°C with

Table 2. Primers used for PCR in this study.

Name of primer	DNA nucleotide sequence[a]
C5	5' CC**GGATCC**TATATCACCTTCGGATTGATGTCC 3' *Bam*H
C3	5' TT**CCCGGG**TGATTTGCTTCGAAAACGCTGACT 3' *Sma*I RBS[b]
D	5' TT**GGATCC**AGTGCTTCGCTTTCTGAGGTTG 3' *Bam*HI RBS[c]
R	5' AA**CCCGGG**AACCTCCGAGAACGCGCGCA 3' *Sma*I

[a]The restriction enzyme sites were introduced as described previously [10]. The modified nucleotides are indicated by bold letters.
[b]The underlined bases indicate the complementary sequences of the ribosome binding site of *mocC*.
[c]The underlined bases indicate the complementary sequences of the ribosome binding site of *mocD*.

shaking. After 4 h, cells were removed by centrifugation and the supernatants were spotted on 3MM filter paper. The filter paper was air-dried, and the remaining substrate in the samples was visualized by the silver nitrate staining method as described previously [4].

β-Galactosidase Assay

Cells were grown to an exponential phase in 3 ml of ATNG medium. Cultures were collected by centrifugation and then washed twice with 1 ml of AT minimal medium. Cells were resuspended in 3 ml of the following media; ATG minimal medium (AT medium containing 0.2% glucose), ATG minimal medium containing 1 mM MOP, or ATG minimal medium containing 1 mM MOP and 0.15% ammonium sulfate. These cells were then grown for another 12 h with aeration, and the resulting β-galactosidase activity was quantified using ONPG as a substrate, as described by Miller [12].

DNA Sequencing Analysis

DNA nucleotide sequences of all DNA fragments amplified by PCR were determined automatically using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, U.S.A.) and ABI PRISM 310 Genetic Analyzer (Perkin Elmer).

Results

Constructions of pMC-*lacZ* and pMD-*lacZ* Fusions

Previous genetic study and DNA nucleotide sequence analysis suggested that the *cis*-acting regulatory elements necessary for the proper expression and regulation of *mocC* and *mocD* were located within 285-bp and 244-bp regions upstream of those genes, respectively [8]. These regions were amplified by PCR as described in Materials and Methods, and the amplified fragments were cloned into the broad-host-range promoter-probe-vector pRG970 [21], to generate transcriptional fusions to the promoterless *lacZ* gene (Fig. 2). The resulting constructions, named pMC-*lacZ* and pMD-*lacZ*, were introduced into *Agrobacterium* strain UIA5, and β-galactosidase activity and β-glucuronidase activity from these strains were examined on ATNG solid media containing X-gal or X-gluc as a substrate. UIA5 harboring pMC-*lacZ* turned blue on plate containing X-gal, and UIA5 harboring pMC-*lacZ* turned blue on plate containing X-gal or X-gluc (data not shown) This suggested that the regions upstream of *mocC*, *mocD*, and *mocR* contain promoters initiating the transcription of the reporter genes.

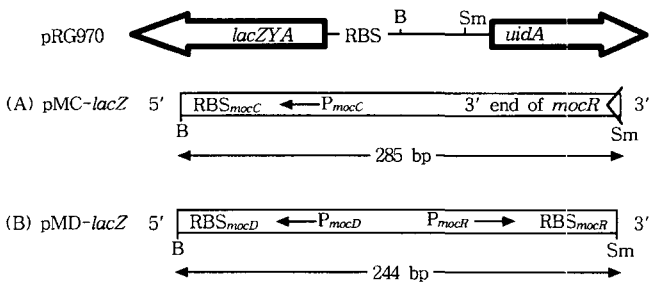

Fig. 2. Construction of pMC-*lacZ* and pMD-*lacZ*.
PCR products containing putative promoter regions upstream of *mocC* and *mocD* were subcloned into the promoter-probe-vector pRG970, to construct pMC-*lacZ* and pMD-*lacZ*, respectively. In pMC-*lacZ*, the fragment containing a ribosome binding site (RBS) and a putative promoter region upstream of *mocC* is transcriptionally fused to promoterless *lacZYA* carrying its own RBS, using the *Bam*HI and *Sma*I sites. In pMD-*lacZ*, the intergenic region between *mocD* and *mocR* is cloned. In the construction, the region containing an RBS and a putative promoter region upstream of *mocD* is transcriptionally fused to a promoterless *lacZYA* carrying its own RBS, and the fragment containing the RBS of *mocR* and its own promoter is translationally fused into the *uidA* lacking its own RBS. Abbreviations: RBS, ribosome binding site; P*mocC*, P*mocD*, and P*mocR*, promoter regions upstream of *mocC*, *mocD*, and *mocR*, respectively; B, *Bam*HI; Sm, *Sma*I.

Cloning of *mocR* and *mocS*

The 2.7-kb *Eco*RI fragment from pYDH208 was subcloned into the *Eco*RI site of pBluescript SK- to construct pBSE22a. The *Sph*I-*Sal*I fragment containing *mocR* from pBSE22a was subcloned into the broad-host-range vector pDSK519 to construct pWH-R (Fig. 3). The 5.7-kb

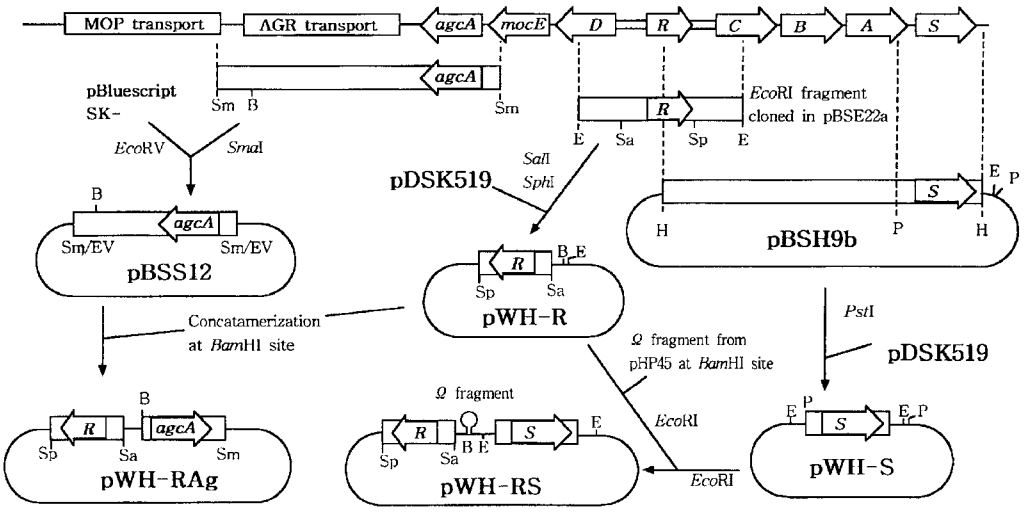

Fig. 3. Construction strategies of various *moc* clones.
The 2.7-kb *Eco*RI fragment 22a containing *mocR* from pYDH208 was subcloned into pBluescript SK- to construct pBSE22a. The *Sph*I-*Sal*I fragment containing *mocR* and its putative promoter region from pBSE22a was subcloned into pDSK519 to construct pWH-R. The 5.7-kb *Hin*dIII fragment from pYDH208 was subcloned into the *Hin*dIII site of pBluescript SK- to construct pBSH9b. The 1.5-kb *Pst*I fragment from pBSH9b was subcloned into pDSK519 to construct pWH-S. Hence, this construction contains the whole *mocS* gene and the region upstream of the gene. The *Eco*RI fragment from pWH-S and the Ω fragment were subcloned into pWH-R to construct pWH-RS. The 5.0-kb *Sma*I fragment containing *agcA* from pYDH208 was subcloned into the *Eco*RV site of pBluescript SK- to construct pBSS12. The *Bam*HI digested pWH-R and pBSS12 were concatamerized to construct pWH-RAg. Abbreviations; E, *Eco*RI; EV, *Eco*RV; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; Sa, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Ω, an omega fragment containing a transcriptional stop signal.

*Hin*dIII fragment from pYDH208 was subcloned into the *Hin*dIII site of pBluescript SK- to construct pBSH9b. The 1.5-kb *Pst*I fragment containing *mocS* from pBSH9b was subcloned into the *Pst*I site of the broad-host-range vector pDSK519 to construct pWH-S. Each construction was introduced into UIA5 containing pMC-*lacZ* and pMD-*lacZ* by a conjugation mediated by S17-1 [18]. The *Eco*RI fragment containing *mocS* from pWH-S and the Ω transcriptional-translational stop signal [16] were subcloned into pWH-R using the *Eco*RI and the *Bam*HI sites to construct pWH-RS. Hence, this plasmid contains both *mocR* and *mocS* oriented divergently, and separated by the transcriptional stop signal so that transcriptions of the genes are not affected by any possible promoters excising somewhere else.

Construction of pWH-4T, a *mot* Operon Clone

To facilitate MOP transport into the tested agrobacterial strains, the 8.8-kb *Bgl*II fragment encoding the MOP transport operon [15] from the library clone pYDH901 [3] was subcloned into the broad-host-range vector pDSK519. From this plasmid, the 5.9-kb fragment containing the whole *mot* operon was cut using the *Sma*I site present just upstream of the operon and the *Xba*I site at the multicloning sites of the vector. The blunt-end of the fragment generated by the *Sma*I-digestion was then ligated with a *Xba*I linker to obtain a *mot* cassette with *Xba*I sites at both ends (the DNA nucleotide sequence of the linker was 5'-TGCTCTAGAGCA-3'). This 5.9-kb *Xba*I fragment containing the whole *mot* genes was subcloned into pRK415 to construct pWH-4T, and the resulting plasmid was introduced into all UIA5 derivatives used in this study for an analysis of the *trans*-acting elements in the regulation of the *moc* operon. To confirm the ability of the *mot* cassette to facilitate MOP transport, pWH-4T was introduced into UIA5(pSaB4::Tn*5*Seq1#221) [8] which carries all the genes necessary for the degradation of MOP, yet lacks the function for MOP transport. The resulting strain, UIA5(pSaB4::Tn*5*Seq1#221, pWH-4T) grew well in the medium containing MOP as a sole carbon source, genetically indicating that the plasmid pWH-4T encodes a functional MOP transport system. To confirm this biochemically, the cells were grown in a liquid medium containing MOP as the sole carbon source. After a three-day incubation, the amount of MOP remaining in the supernatant was examined as described in Materials and Methods. Comparing the supernatant grown with cells lacking pWH-4T as a negative control, the amount of MOP was significantly reduced (data not shown). This result suggests that the pWH-4T plasmid confers the opine transport function on the cells.

Effect of MocR on the Expression of *mocC* and *mocD*

β-Galactosidase activities were measured from UIA5 harboring pWH-R and each of pMC-*lacZ* and pMD-*lacZ* in the presence or absence of MOP. Without the *mocR* subclone, the β-galactosidase activities from the pMC-*lacZ* fusion and the pMD-*lacZ* fusion were more than 2,000 Miller units. However, regardless of whether MOP

Table 3. Galactosidase activity of *Agrobacterium* strain UIA5 harboring the pMC-*lacZ* fusion or pMD-*lacZ* fusion introduced with a *mocR* (pWH-R), *mocS* (pWH-S), or *mocR* and *mocS* subclone (pWH-RS).

Strain (plasmid)[a]	β-Galactosidase activity (Miller unit)[b]	
	-MOP	+MOP[c]
UIA5(pRG970, pDSK519, pWH-4T)	8	5
UIA5(pRG970, pWH-R, pWH-4T)	20	16
UIA5(pRG970, pWH-S, pWH-4T)	23	0
UIA5(pRG970, pWH-RS, pWH-4T)	0	0
UIA5(pMC-*lacZ*, pDSK519, pWH-4T)	2640	3621
UIA5(pMC-*lacZ*, pWH-R, pWH-4T)	66	98
UIA5(pMC-*lacZ*, pWH-S, pWH-4T)	2058	2676
UIA5(pMC-*lacZ*, pWH-RS, pWH-4T)	13	29
UIA5(pMD-*lacZ*, pDSK519, pWH-4T)	2491	2383
UIA5(pMD-*lacZ*, pWH-R, pWH-4T)	30	44
UIA5(pMD-*lacZ*, pWH-S, pWH-4T)	6660	6826
UIA5(pMD-*lacZ*, pWH-RS, pWH-4T)	12	0

[a]All the strains used in this study were introduced with pWH-4T, a clone containing the MOP transport genes from pTi15955, to facilitate the transport of MOP.
[b]Cells were grown in an ATN minimal medium containing 0.2% glucose as the sole carbon source. The β-galactosidase activity assay was carried out as described in Materials and Methods.
[c]MOP at a final concentration of 1 mM was added for induction.

was supplemented or not, the expression of each fusion was completely repressed by pWH-R (Table 3). The addition of $(NH_4)_2SO_4$ as a nitrogen source did not significantly affect the level of repression in these conditions (data not shown). These results suggest that MocR represses the expression of *mocC* and *mocD*, and that MOP is not an inducer for the expression of the *moc* operons.

Effect of MocS on the Expression of *mocC* and *mocD*

The plasmid pWH-S, which encodes MocS, slightly repressed the expression of β-galactosidase from pMC-*lacZ*, yet did not repress that from pMD-*lacZ*, regardless of whether MOP was supplemented or not (Table 3). The plasmid pWH-RS, which encodes for MocR and MocS, repressed the expression of pMC-*lacZ* and pMD-*lacZ* regardless of whether MOP was supplemented or not. The β-galactosidase activities from the two reporters appeared to be lower in the presence of the two repressors when compared to those in the presence of MocR alone. This result suggests that MocS is not responsible for a tight repression of the expressions of *mocC*, *mocD*, and *mocR*, and only exerts a weak repression of the genes.

AGR Induces the Expression of *mocC* and *mocD*

As shown above, MOP could not relieve the repression of *mocC* and *mocD* mediated by MocR. In order to elucidate

Table 4. β-Galactosidase activities from derivatives of UIA5 harboring the pMC-*lacZ* fusion or pMD-*lacZ* fusion.

Harbored plasmids[a]	Medium compositions[b]		β-Galactosidase activity[e]
	Addition of MOP[c]	Addition of $(NH_4)_2SO_4$[d]	
pMC-*lacZ*, pDSK519	-	-	2519
	-	+	2715
	+	-	3339
	+	+	4125
pMC-*lacZ*, pWH-R	-	-	19
	-	+	16
	+	-	16
	+	+	15
pMC-*lacZ*, pWH-RAg	-	-	29
	-	+	36
	+	-	189
	+	+	198
pMD-*lacZ*, pDSK519	-	-	3365
	-	+	3385
	+	-	3558
	+	+	3293
pMD-*lacZ*, pWH-R	-	-	11
	-	+	15
	+	-	15
	+	+	11
pMD-*lacZ*, pWH-RAg	-	-	28
	-	+	38
	+	-	185
	+	+	186

[a]All the strains used in this study contained the pWH-4T plasmid, a MOP transport clone, to facillitate the transport of MOP.
[b]AT minimal medium containing 0.2% glucose was used to grow the cells.
[c]MOP was added at a final concentration of 1 mM.
[d]Final concentration was 0.15%.
[e]Miller units.

a genuine inducer in the regulation of the operon, catabolic intermediates possibly generated in the MOP catabolism, such as SOP, glutamine, glucose, and mannose, were tested for their ability to induce the expression of the operon. To test whether SOP, the first catabolic intermediate of MOP [8], is an inducer, pDSK509::325B [8] that encodes *mocR* and *mocC* was introduced into UIA5 containing either of pMC-*lacZ* and pMD-*lacZ* fusions, and MOP was supplemented in the medium. The levels of β-galactosidase from the resulting strains were not induced in the presence of MOP (data not shown). The addition of mannose, glucose, or glutamine into the medium, instead of MOP, also did not induce the expression of both *lacZ* fusions (data not shown). These results suggested that none of the tested

catabolic intermediates of MOP - SOP, glutamine, glucose, or mannose - are inducers of the *moc* operon. Previous studies using wild-type MOP-type agrobacterial strains apparently indicated that the catabolism of MOP was induced by the addition of MOP. The ability of an agrobacterial strain containing pYDH208 to utilize MOP was also induced when MOP was added into the media [5]. All of these strains contain an *agcA* gene, which encodes for MOP cyclase which catalyzes the lactonization of MOP to AGR [6]. Therefore, it is highly possible that when MOP is added, the opine is converted to AGR by the enzyme in those strains, and the product may then induce the expression of the *moc* genes. In order to verify this hypothesis, pWH-RAg, which contains *mocR* and *agcA*, was constructed. Hence, in cells harboring this plasmid, transported MOP is converted to SOP by oxidation activity of MOP oxidoreductase encoded by *mocC*. This plasmid was introduced into UIA5 harboring pWH-4T and either of pMC-*lacZ* and pMD-*lacZ*, and the β-galactosidase activities of the resulting strains were measured. The expression of β-galactosidase from the strains was induced by the addition of MOP. The expression level was more than six-times than that in the addition of glucose only (Table 4). This result shows that AGR, not MOP, is a natural inducer for the expression of *moc* operons.

Effect of Inorganic Nitrogen on the Induction of *mocC* and *mocD*

We tested the effect of inorganic nitrogen on the induction levels of *mocC* and *mocD*. The β-galactosidase activities of UIA5 cells harboring pWH-RAg and either of pMC-*lacZ* and pMD-*lacZ* were measured after grown in MOP and in the presence or absence of ammonium sulfate as a nitrogen source. As shown in Table 4, the supplement of ammonium sulfate did not exert a significant effect on the induction level, suggesting that the inorganic nitrogen does not affect the expression of the genes in our reporter system.

DISCUSSION

Catabolism of mannityl opines conferred by the octopine type-Ti plasmid pTi15955 of *Agrobacterium tumefaciens* 15955 have been intensively studied both biochemically and molecular genetically [3, 5, 6, 8, 9]. Nevertheless, little is known on how these genes for the utilization of the opines are controlled in the response to the presence of various carbon and nitrogen sources. This is partly due to the complexity in the organization of the genes for the utilization of those opines. The information accumulated to-date shows that at least four different ABC-type transport systems for the four mannityl opines are present in the Ti plasmid, and each transport system is encoded by three to four genes organized as operons and is associated with the transport of each of the mannityl opines. It appears that at least four genes and at least three genes are involved in the degradation of AGR and MOP and of MOA and AGA, respectively [9, 17]. Considering the similarity in the chemical structures of the four opines, it is likely that a very sophisticated regulation of the expressions of the operons for the opines is necessary for the economical management of the energy for the substrate utilization. Furthermore, since mannityl opines comprise nitrogen atoms, it is also possible that the expression of the catabolism genes may be modulated by the intracellular level of nitrogen. The situation is even more complicated when considering the recent findings showing that the cryptic plasmid, pAtC58, present in the NT1 strain encodes functions for the transport of MOP and its oxidized product, SOP, along with functions for the SOP catabolism [ref. 20 and our unpublished data]. Therefore, it is expected that the expression of the genes for the utilization of the opines is controlled in a harmonious and delicate manner. To overcome the complexity in studying this regulation, each of the putative regulators were individually cloned, and each of the regions upstream of the first genes of the *moc* operons was fused to a promoterless *lacZ* as a reporter gene. The *lacZ* fusions were co-introduced to cells with each of the putative repressor clones, and the resulting β-galactosidase activities were measured under various conditions. The deduced amino acid sequences of *mocR* and *mocS* showed high similarities with various repressors in sugar-utilizing operons. Hence, it was assumed that these two genes code for the negative-regulators in *moc* operons. It was also assumed that the regions upstream of *mocC* and *mocD* contain the *cis*-acting elements necessary for the proper expression and regulation of the divergently oriented operons. Even though *mocR* precedes *mocC* in its position, the region upstream of *mocC* appears to have its own promoter. This assumption is based on the observation that an insertion mutation in *mocR* did not cause a polar effect on *mocC* (data not shown). All the experiments were performed using the agrobacterial strain UIA5 as the host strain. This strain lacks any cognate plasmid, accordingly, any possible effects caused by factors encoded by a cryptic plasmid can be excluded.

This study showed that MocR severely represses the expression of *mocC* and *mocD*. A previous study by Hong *et al.* [5] using NT1 harboring pYDH208 with *lacZ* fusions in *mocC* and *mocD* generated by a Tn*3*HoHo1 insertion showed that the β-galactosidase activities from the fusions were induced by the addition of MOP in the medium. This study led the authors to assume that MOP may be an inducer for the expression of the *moc* operon. However, in this study, the expression of those genes was not induced even with the addition of MOP. Other possible catabolic intermediates of MOP such as SOP, glutamine, glucose,

and mannose also failed to induce the gene expression. However, when an *agcA* subclone was introduced, the expression of *mocC* and *mocD* became derepressed in the presence of MOP. These results suggest that AGR, which is cyclized from MOP by MOP cyclase encoded by *agcA*, is a genuine inducer for the expression of, at least, *mocC* and *mocD*. Therefore, a reinterpretation of the results of the previous study can explain that the β-galactosidase activities of the fusions in pYDH208 must have been induced by AGR converted from MOP by the MOP cyclase present in the cosmid clone. Even though the β-galactosidase activities of UIA5 containing pWH-RAg and either of pMC-*lacZ* and pMD-*lacZ* were induced by MOP, the Miller unit values were much lower than those of UIA5 harboring the reporters but lacking the *mocR* clone. This may be due to the copy number of the incomplete pWH-RAg plasmid. In wild-type strain 15955, there exists only one copy of *mocR*, that is encoded on the Ti plasmid. It is likely that the high copy number of pWH-RAg resulted in an incomplete derepression.

MocS, which has a close similarity with MocR in the predicted amino acid sequences, did not significantly repress the expression of *mocC* and *mocD*. The β-galactosidase activity from the *lacZ* fusion was slightly reduced by the presence of MocS. This result suggests that MocS is not responsible for a tight repression of the *moc* operon. This result, however, creates a dilemma in interpreting the previous study which showed that NT1 harboring pYDH208 with a transposon inserted in *mocS* grew faster than did NT1 harboring wild-type pYDH208 on medium containing MOP [5]. It is possible that MocS plays a role as a weak repressor for *moc* genes, and a mutation in this gene results in the enhanced growth on MOP. Another possibility is that the cryptic plasmid pAtC58 in strain NT1 used in that study may have some relationships with such a phenotype.

The *mocS* gene is located right next to the region which is associated with the utilization of two other mannityl opines, MOA and AGA [7]. Hence, it is possible that the main role of MocS is as a regulator of the genes for these opines. Sangbom *et al.* [17] recently studied genes for utilization of mannopinic acid (MOA) and agropinic acid (AGA) mapping to the region adjacent to the *moc* operons. They showed that the repressor, *moaR*, in the region is responsible for the negative-regulation of those genes. The cosmid clones they used for the study however, did not contain a *mocS* region. Therefore, the role of *mocS* in the regulation of those operons is still unclear, and the exact role of *mocS* remains to be elucidated.

We examined the effect of inorganic nitrogen on the expression of *mocC* and *mocD*. When MOP and nitrogen were added together into the medium, the induction level of *mocC* and *mocD* from cells harboring an *agcA* clone was not significantly different from that when MOP alone is supplied. This result is contradictory to the previous report [5] showing that the β-galactosidase activity from NT1 cells harboring pYDH208 with *lacZ* fusion generated by Tn*3*HoHo1 insertion on *mocC* or *mocD* was significantly reduced when nitrogen sulfate was supplemented in addition to MOP. Unlike in our study, this experiment was performed using NT1 as a host strain. Moreover, the *lacZ*-fusion was generated in the pYDH208 plasmid that contains a 21-kb fragment comprising MOP and AGR transport systems and all the *moc* genes. It is likely that the nitrogen-dependent modulation shown in the study of Hong and his colleagues was due to some effects from functions encoded either by the pYDH208 plasmid or by the cryptic plasmid pAtC58 in NT1. Considering that each molecule of MOP and AGR contains two nitrogen atoms, the supply of an extra nitrogen source may affect the expression of the genes for the opine utilization. Our study apparently showed that a nitrogen-dependent modulation of the *moc* genes is not mediated by MocR-associated regulation. Functions responsible for the nitrogen-dependent regulation remains to be elucidated.

Future studies will be focused on the exact role of *mocS* and the nitrogen-dependent regulation of *moc* operons. In addition, it may be valuable to investigate the *cis*-acting elements present upstream of *mocC* and *mocD* associated with regulation of the operons.

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