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Genes for the Catabolism of Deoxyfructosyl Glutamine in pAtC58 Are Attributed to Utilization of Octopine in *Agrobacterium tumefaciens* Strain NT1

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Abstract Nopaline-type *Agrobacterium tumefaciens* strain C58 cannot utilize octopine (Oct) as the sole carbon and nitrogen sources. This strain harbors two plasmids; a virulent plasmid, pTiC58, and a megaplasmid, pAtC58. From strain NT1, which is a derivative of C58 harboring only pAtC58, we isolated spontaneous mutants that utilize Oct as the sole nitrogen source. These Oct-catabolizing mutants, however, could not utilize the opine as the sole carbon source. In contrast, strain UIA5, a plasmid-free derivative of C58, could not give rise to such mutants. The mutations isolated from NT1 were mapped to *socR* in pAtC58, which is a negative regulator of the *soc* operon responsible for the uptake and catabolism of an Amadori opine, deoxyfructosyl glutamine (Dfg). A derivative of UIA5 carrying a clone of the *soc* operon with a transposon inserted in *socR* also utilizes Oct as the sole nitrogen source. However, UIA5 harboring the operon with mutations in each of the structural genes in the *soc* operon, *socA*, *B*, *C*, and *D*, lost the ability to generate spontaneous Oct-utilizing mutants, suggesting that *soc* genes in pAtC58 are required for the utilization of Oct as a nitrogen source, and that derepressed expression of these genes allows cells to utilize Oct. In contrast, Oct-catabolizing mutants derived from C58, which grew using Oct as the sole nitrogen source, could also utilize the opine as the sole carbon source. These mutants did not carry any detectable mutations in *socR* or the region upstream to the gene in pAtC58, suggesting that mutations occurring elsewhere in the genome, most likely in pTiC58, allow the uptake and catabolism of the opine.

Key words: *Agrobacterium tumefaciens*, octopine, deoxyfructosyl glutamine, pAtC58

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Agrobacterium tumefaciens spp. induce plant tumors called crown galls in dicotyledonous plants in nature [29]. The pathogens harbor virulent plasmids, called Ti (tumor-inducing) plasmids. A part of the plasmid, called T-DNA, is transferred into plant cells upon infection, and oncogenes encoded by the region are expressed from the plant-specific promoter, causing the infected plant cells to become cancerous [25]. The T-DNA also encodes a set of genes responsible for the synthesis of opines. Opine synthesized from plant tumor cells diffuses out to the soil, and is taken up and catabolized by functions encoded by Ti plasmid in *A. tumefaciens* [7], which serves as sources of carbon and nitrogen for *A. tumefaciens*. Up to now, more than twenty different types of opines have been found. The type of opine a Ti plasmid induces the synthesis in plant cells which is generally the same as the type of opine the Ti plasmid allows to catabolize. 'Opine concept' has been proposed to rationalize this one-to-one relationship between the type of opine an *A. tumefaciens* spp. synthesizes and the type of opine the pathogen utilizes [7, 19]. This hypothesis suggests that *A. tumefaciens* spp. bioengineers plant cells to produce opine which can serve as sources of carbon, nitrogen, and energy for the pathogen. This hypothesis has been experimentally verified by several groups [10, 18, 22, 28].

Nopaline-type *A. tumefaciens* strain C58 can induce plant tumors producing nopaline (Nop) and agrocinopines [19]. The genes for opine biosynthesis are encoded by the T-region of Ti plasmid, pTiC58, which also encodes genes for the uptake and catabolism of these opines in the non T-DNA region. Another type of opine also catabolized by this pathogen is an Amadori compound, deoxyfructosyl glutamine (Dfg) (8, 27; Fig. 1). The genes for utilization of this Amadori opine are encoded not by pTiC58 but by a megaplasmid in the strain, called pAtC58 [3, 9]. The

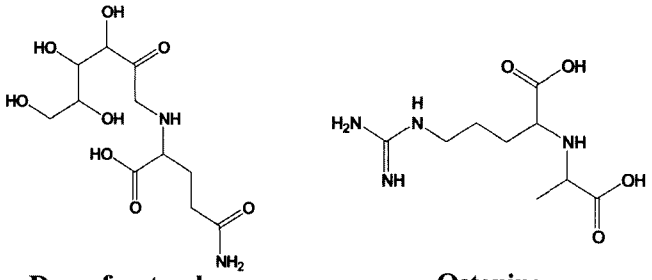

Fig. 1. Chemical structures of Dfg and Oct.

operon responsible for the utilization of Dfg is composed of five genes: one regulatory gene, *socR*, and four structural genes *socA*, *B*, *C*, and *D* (3; Fig. 2). SocC and D are oxidoreductase and fructosyl-amino acid oxidase, respectively, which are believed to be responsible for the degradation of Dfg into fructose and glutamine [3]. SocA and B are components of an ABC-type transporter, and they also are responsible for the transportation of mannopine (MOP) as well as its genuine substrate Dfg in the strain C58 [3]. These four structural genes are negatively regulated by SocR, which is a MocR homolog [12], and Dfg or its catabolic intermediate is an inducer [3]. We proposed that *soc* genes are not specific for the degradation of opine, but rather are involved in utilization of Amadori compounds spontaneously generated in soil environments and in rotting plant materials [3]. In accordance with this idea, *soc* genes are phylogenically distant from genes for the utilization of chemically related opine, mannityl opines, suggesting that these two gene sets are evolved independently (unpublished results).

A previous report demonstrated that mutations in inborn megaplasmid pAtC58 allow the nopaline-type *A. tumefaciens* strain C58 to utilize octopine (Oct; Fig. 1) as the sole nitrogen source [26]. This report led us to consider the possibility that the *soc* genes in pAtC58, which apparently are the gene set associated with opine catabolim in pAtC58 based on the genome sequence analysis [9], could be responsible for the Oct-utilization phenotype of the strain.

In this study, we determined gene(s) responsible for utilization of Oct in the Nop-type strain C58 through genetic analyses of spontaneous Oct-utilizing mutants, and showed that *soc* genes were absolutely necessary for the generation of spontaneous mutants which can utilize Oct as the sole nitrogen source. In addition, we also showed that some mutations in the genome of C58 outside of pAtC58, most likely in pTiC58, also allow the strain to utilize the opine as a source of not only nitrogen but also carbon.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. A derivative of pAtC58, pAtC58Δ*socR*, which has a deletion in *socR*, was constructed by allelic exchange as described previously [4, 17].

Culture Media, Growth Conditions, and Chemicals

Nutrient broth (NB) (Difco Laboratories, Detroits, MI, U.S.A.) and Luria-Bertani broth (LB) (Difco Laboratories) were

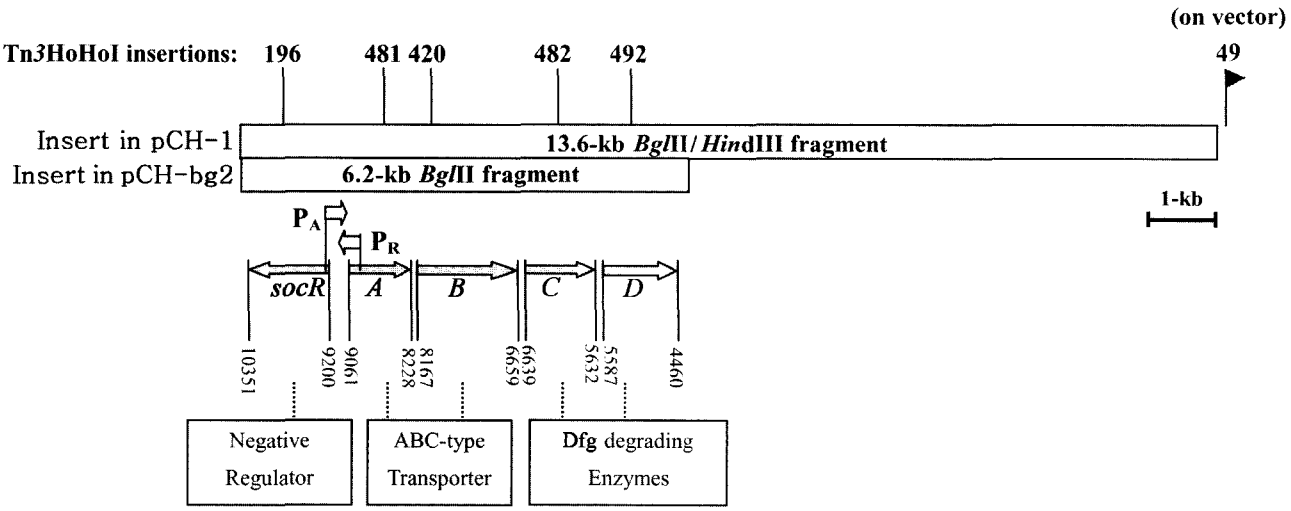

Fig. 2. Genetic map of the region of pAtC58 containing the *soc* operon and subclones.
The positions of the transposon insertions are indicated by the vertical lines. The positions of the *soc* genes are indicated by shadow arrows, and the positions and orientations of promoters are denoted by vertical arrows and open arrows. The numbers below the *soc* genes represent the coordinates of the region with respect to the complete nucleotide sequences of pAgC58 in the GenBank database under accession number AE007872 [9].

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

Strains and plasmids	Relevant genotype and characteristics[a]	Source or reference
Strains		
C58	Wild-type isolate; pTiC58$^+$, pAtC58$^+$, Oct$^-$	Our collection
C58-OCM[b]	Spontaneous octopine-utilizing mutant (OCM) derived from C58	This study
NT1	pTiC58$^-$, pAtC58$^+$ derivative of C58, Oct$^-$	Our collection
NT1-OCM	Spontaneous octopine-utilizing mutant derived from NT1	This study
NT1ΔR	A derivative of NT1 harboring pAtC58Δ*socR*, Oct$^+$	3
UIA5	pTiC58$^-$, pAtC58$^-$ derivative of the C58, Oct$^-$; Rif^r Sm^r	Our collection
UIA5 (pCH-Bg2)-OCM	Spontaneous octopine-utilizing mutant derived from UIA5 (pCH-Bg2); Tc^r	This study
Plasmids		
pAtC58Δ*socR*	A *socR*-deletion derivative of pAtC58	3
pBS-Bg2	A clone containing the *soc* operon	3
pBS-Bg2ND83	A clone containing *socR* derived from pBS-Bg2; Ap^r	3
pCHAt56	Cosmid clone of pAtC58 containing the whole *soc* operon; Tc^r	3
pCH-Bg2	Derivative of pRK415 containing a 6.2-kb *Bgl*II fragment 2 from pCHAt56	3
pCH-1	Derivative of pRK415 containing a 13.6-kb *Blg*II-*Hin*dIII fragment from pCHAt56; Tc^r	3
pCH-Bg2^{OCM}	Plasmid isolated from UIA5 (pCH-Bg2)-OCM	This study
pCH-Bg2ΔR	Derivative of pCH-Bg2 with a deletion in *socR*; Tc^r	3
pRK415	Broad-host range IncP cloning vector; Tc^r	14
pDSK519	Broad-host-range IncQ cloning vector; Km^r	14
pDSK-*socR*	*socR* clone, a concatemer of pBS-Bg2ND83 and pDSK519; Cb^r Km^r	This study

[a]Oct$^+$, utilizes Oct as the sole nitrogen source; Oct$^-$, cannot utilize Oct as the sole carbon and nitrogen sources.

[b]This mutant utilizes octopine as the sole carbon and nitrogen sources.

Abbreviations: Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance.

used as rich media for *A. tumefaciens* and *Escherichia coli*, respectively. AT minimal medium [19] supplemented with 0.15% $(NH_4)_2SO_4$ and 0.2% glucose (ATNG) was used as minimal media for *A. tumefaciens* [3]. AT minimal medium supplemented with 0.15% $(NH_4)_2SO_4$ and 5 mM Oct (ATNO) was used to test strains of *A. tumefaciens* for the ability to utilize Oct as the sole carbon source. AT minimal medium supplemented with 0.1% mannitol and 0.05% Oct (ATMO) was used to test strains of *A. tumefaciens* for the ability to utilize Oct as the sole nitrogen source. Noble agar (Difco Laboratories, final concentration 1.8%) was used to solidify the minimal medium. Strains of *A. tumefaciens* were grown at 28°C, while strains of *E. coli* were grown at 37°C. For *A. tumefaciens*, carbenicillin (Cb) at 100 μg/ml, kanamycin (Km) at 100 μg/ml, and tetracycline (Tc) at 2 μg/ml were used. For *E. coli*, ampicillin (Ap) at 100 μg/ml, Km at 10 μg/ml, and Tc at 10 μg/ml were used. All antibiotics, mannopine (Mop), nopaline (Nop), and Oct were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Dfg was chemically synthesized as described previously [6]. Mannopinic acid (Moa), Chrysopine (Chr), and deoxyfructosyloxoproline (Dfop) were kindly provided by William S. Chilton (North Carolina State University, U.S.A.).

DNA Manipulation and Transformation

Isolations of plasmid DNA from *Agrobacterium* cells were performed by a rapid alkaline lysis procedure as described previously [3]. Restriction enzyme digestions and ligations were carried out as recommended by the manufacturer (Promega, Madison, WI, U.S.A.). Transformation of *A. tumefaciens* strains and *E. coli* was performed as previously described [3, 21].

Oct Utilization Test

Growth was assessed by daily visual inspection of colonies grown on solid ATNO and ATMO media for a two-week period.

Nucleotide Sequence Analysis

Nucleotide sequences were determined using an ABI-373A automated DNA sequencer (Perkin Elmer Corp., Foster City, CA, U.S.A.). Nucleotide and amino acid sequences were compared to those in the GenBank database using the BLASTA algorithm [1].

RESULTS

Generation of Spontaneous Oct-Catabolizing Mutants (OCM) from Derivatives of Nop-Type *A. tumefaciens* Strain C58

We tested Nop-type *A. tumefaciens* strain C58 and its derivatives for the capability to give rise to spontaneous mutants, which can grow by utilizing Oct as the sole

source of carbon or nitrogen. All of the strains tested showed a basal level of growth on solid ATMO media lacking a nitrogen supplement probably due to the capability of *Agrobacterium* spp. to fix nitrogen at low efficiency [13]. However, some of the tested strains gave rise to mutants which grew much faster than other cells on the medium. Strain NT1, a derivative of C58 containing pAtC58 but lacking pTiC58, gave rise to such spontaneous mutants which grew faster using Oct as the sole nitrogen source (Table 2). In contrast, UIA5, a pTiC58$^-$ pAtC58$^-$ derivative of C58, could not generate fast-growing mutants on solid ATMO medium. These results suggest that some genes encoded by pAtC58 are responsible for generating spontaneous mutants which grow faster using Oct as the sole nitrogen source. NT1ΔR, which is a derivative of NT1 harboring pAtC58 with a null-mutation in *socR* [3], can naturally grow faster than wild-type NT1 using Oct as the sole nitrogen source, suggesting that the *soc* operon is able to utilize Oct. When pCHAt56, which is a cosmid clone of pAtC58 containing the whole *soc* operon, was introduced into UIA5, the resulting cells obtained ability to give rise to fast-growing spontaneous mutants on ATMO. Two constructions, pCH-1 and pCH-Bg2 (Fig. 2), which are subclones of pCHAt56 containing the whole *soc* operon, also allowed UIA5 to generate such fast-growing mutants on ATMO, whereas the vector pRK415 could not (Table 2). These results suggested that functions encoded by the *soc* operon are responsible for the ability of cells to utilize Oct as the sole nitrogen source.

From one of the spontaneous fast-growing mutants from UIA5(pCH-Bg2) obtained on ATMO medium, the plasmid named pCH-Bg2^{OCM} was isolated and introduced into wild-type UIA5. The resulting strain, UIA5(pCH-Bg2^{OCM}), grew as fast as the parental spontaneous fast-growing mutant (Table 2), suggesting that the mutation in the parental mutant occurred in the pCH-Bg2^{OCM} plasmid. It was speculated that the mutation may occur in *socR*, which is a negative regulator in the *soc* operon [3, 12]. Supporting this hypothesis, UIA5 harboring pCH-Bg2ΔR [3], which is a derivative of pCH-Bg2 with a deletion in *socR*, grew as

Table 2. The capability of derivatives of Nop-type *A. tumefaciens* strain C58 to generate mutants, which can utilize Oct as the sole sources of carbon or nitrogen.

Strains	Occurrence of the OCM[a]	
	Type I	Type II
C58	+	+
NT1	-	+
NT1ΔR	-	NA[b]
UIA5	-	-
UIA5 (pRK415)	-	-
UIA5 (pCHAt56)	-	+
UIA5 (pCH-1)	-	+
UIA5 (pCH-Bg2)	-	+
UIA5 (pCH-Bg2^{OCM})[c]	-	NA[b]
UIA5 (pCH-Bg2*ΔR*)	-	NA[b]

[a]Tested strains were grown in solidified AT minimal medium supplemented with 0.1% mannitol and 0.05% octopine as the sole carbon and nitrogen source, respectively. Cultures were observed daily for a two-week period. Growth was recorded as following scales: -, no generation of spontaneous OCM; +, gives rise to OCMs; NA (not applicable). OCMs obtained were then tested for the ability to utilize Oct as the sole carbon or nitrogen sources. Type I mutant can utilize Oct as the sole source of carbon as well as nitrogen. Type II mutant can utilize Oct only as the sole nitrogen source.
[b]These strains naturally grew using Oct as the sole nitrogen source.
[c]A derivative of UIA5 introduced with the plasmid pCH-Bg2^{OCM}, which was isolated from UIA5 (pCH-Bg2^{OCM}).

Table 3. The capability of NT1-OCM to utilize Oct as a nitrogen source, but not that of C58-OCM, was abolished by in-trans supply of SocR.

Strain (resident plasmids)	Growth utilizing Oct[a]	
	as sole carbon source (on ATNO medium)	as sole nitrogen source (on ATMO medium)
UIA5 (pCH-Bg2*ΔR*)	-	+
UIA5 (pCH-Bg2*ΔR*, pDSK519)	-	+
UIA5 (pCH-Bg2*ΔR*, pDSK-*socR*)	-	-
C58-OCM typeI	+	+
C58-OCM typeII	-	+
NT1-OCM	-	+
UIA5 (pCH-Bg2^{OCM})[b]	-	+
C58-OCM typeI (pDSK-*socR*)	+	+
C58-OCM typeII (pDSK-*socR*)	-	+
NT1-OCM (pDSK-*socR*)	-	-
UIA5 (pCH-Bg2^{OCM}, pDSK-*socR*)[b]	-	-

[a]Strains were tested on ATNO and ATMO media. ATNO medium is AT minimal medium supplemented with 0.15% ammonium sulfate and 5 mM Oct as the sole sources of nitrogen and carbon, respectively. ATMO medium was prepared by AT minimal medium supplemented with 0.1% mannitol and 0.05% Oct as the sole carbon and nitrogen sources, respectively. Growth was observed daily for a two-week period, and recorded as the following scales: -, no growth and +, good growth.
[b]Seven independent mutants were tested, and all of the tested mutants showed the same phenotype.

fast as the spontaneous fast-growing mutant on solid ATMO medium (Table 2).

All of the fast-growing mutants obtained from derivatives of NT1 and UIA5 shown in Table 2 were also tested for their ability to utilize Oct as the sole carbon source. None of these mutants could grow using the opine as the sole carbon source. The wild-type C58 strain also gave rise to spontaneous fast-growing mutants on solid ATMO medium. In contrast to OCMs obtained from NT1, those mutants generated from C58, named C58-OCM, could be divided into two groups: one group named type-I C58-OCM could also utilize Oct as the sole carbon source, but the other named type-II C58-OCM could not (Table 2).

A *socR* Clone Suppresses the Ability of the Fast-Growing Mutants Derived from NT1 to Utilize Oct as the Sole Nitrogen Source

When a *socR* clone was introduced into NT1-OCM, the resulting transformants grew slowly on ATMO medium and did not generate fast-growing mutants on the medium (Table 3). Similarly, fast-growing mutants from UIA5 (pCH-Bg2ΔR) and UIA5(pCH-Bg2^{OCM}) introduced with a *socR* clone grew slowly on ATMO medium and did not generate fast-growing mutants. In contrast, both of the two types of fast-growing mutants derived from C58 still grew fast on solid ATMO medium even after being introduced with a *socR* clone.

All the *soc* Genes for Utilization of Dfg are Required for Generation of Oct-Utilizing Fast-Growing Mutants

Consistent with the above results, UIA5 harboring pCH-1::Tn*3*HoHo1#196, which has a transposon inserted in *socR*, also grew as fast as the fast-growing mutants, but UIA5 harboring pCH-1::Tn*3*HoHo1#49 that has an insertion in the vector portion grew slowly and generated fast-growing mutants on ATMO. However, UIA5 carrying pCH-1 with insertions in any of the *soc* genes failed to give rise to such mutants, suggesting that functional products of all *soc* are required for the fast-growing phenotype (Table 4).

Table 4. The capability to generate OCM of UIA5 harboring the *soc* operon with a transposon insertion in various *soc* genes.

Strain	Position of insertion[a]	Occurrence of OCM
UIA5 (pCH-1::Tn*3*HoHoI#196)	*socR*	NA[b]
UIA5 (pCH-1::Tn*3*HoHoI#481)	*socA*	-
UIA5 (pCH-1::Tn*3*HoHoI#420)	*socB*	-
UIA5 (pCH-1::Tn*3*HoHoI#482)	*socC*	-
UIA5 (pCH-1::Tn*3*HoHoI#492)	*socD*	-
UIA5 (pCH-1::Tn*3*HoHoI#49)	vector	+

[a]Fine map of the insertions was reported previously [3].

[b]NA, not applicable. This strain grew well on solid ATMO medium.

DNA Sequence Analysis of *socR* Genes from the OCMs

We determined the DNA nucleotide sequences of *socR* genes from OCMs obtained from derivatives of C58, NT1, and UIA5 strains. OCMs derived from C58 (C58-OCM, type I and type II) do not have any mutations in *socR* genes of pAtC58. However, independent clones of OCMs from NT1 and UIA5(pCH-Bg2) carry mutations in the base C at the position of the coordinate number 10203 (the GenBank database accession number, AE007872) in *socR* genes, which were transversed to the base G. These results suggested that derepressed expression of *soc* genes is responsible for the generation of OCM from NT1 and UIA5(pCH-Bg2)

Discussion

Opines have attracted the special attentions of microbiologists in recent decades due to the unique ecological and physiological roles in the inter-Kingdom relationships between *A. tumefaciens* and plants. The 'Opine Concept' [19] described that pathogenic agrobacterial strains bioengineer plant cells to produce opine in large quantity, which can be utilized by the pathogen as a food. This hypothesis was based upon the observation that the type of opines produced by plant tumor cells 'generally' matches with the type of opines utilized by the inducing *Agrobacterium* strain. For instance, Nop-type *A. tumefaciens* strain C58 induces plant tumor cells producing Nop and agrocinopines, and these opines can be utilized by C58. However, not all the opines that an agrobacterial strain can utilize are produced by plant tumor cells induced by the strain. In other words, *Agrobacterium* spp. carry the ability to utilize more types of opines than those which are produced by plant tumor cells they induced.

Dfg is an Amadori compound, which is produced by plant tumor cells induced by a few known *A. tumefaciens* strains such as chrysopine-type strains and fig-strains [6, 27]. However, this Amadori compound can also be utilized by numerous *Agrobacterium* strains including nopaline-type strain C58, octopine-type strains 15955 and R10, mannityl opine-type strain Bo542, *A. vitis* strains, and *A. rhizogenes* strain 8196 [3]. Furthermore, Amadori compounds can be utilized even by some phylogenically distant soil microbes such as *Pseudomonas* spp. [23], *Corynebacterium* sp. [11], *Candida* sp. [20], and *Aspergillus* sp. [24]. Organic compounds either spontaneously formed or produced by plants affect the dynamics of bacterial community structures in rhizosphere [15, 16]. In keeping with the observation that Amadori compounds can be spontaneously generated from sugar and amino acids in nature [2], it is proposed that the ability to utilize Dfg is widely dispersed among soil bacteria [3]. In this regard, it is possible that *soc* genes responsible for the utilization of Dfg have evolved for

utilization of Dfg not as an opine but rather as one of compounds spontaneously generated in soil.

In this study, we showed that Soc functions are responsible for the utilization of Oct as the sole nitrogen source, but only when the expression is derepressed. We previously showed that SocCD can take up a structurally related opine Mop [3]. This suggests that Soc functions have broad substrate specificities, and the substrate specificity of the *soc* operon is determined by the negative regulator SocR. The biochemical basis of the way in which Soc functions to utilize Oct as the sole nitrogen source but not as the carbon source remain to be elucidated. However, this study clearly shows that Soc functions are necessary for the generation of OCM from NT1. The biochemical reactions responsible for this utilization also remain to be elucidated.

OCM obtained from C58 is clearly distinct from those from NT1. The mutants could utilize Oct as a source of carbon as well as nitrogen. Furthermore, unlike OCM-NT1, none of OCM-C58 carries mutations in *socR* of pAtC58, and extended examination of nucleotide sequences of the *soc* operon did not reveal any occurrence of mutations (data not shown), suggesting that OCM-C58 carries mutations elsewhere in the genome, possibly in the Ti plasmid. These observations may suggest that the genome of C58 carries at least more than two determinants which allow the utilization of octopine after generations of mutation.

The results obtained in this study suggest a complexity of genes associated with opines. In a given *Agrobacterium* strain, numerous genes are present which are involved in related opine compounds and are potentially able to utilize some distantly related opines after mutations [5]. Some of these genes may represent a possible ancestral form of genes from which gene sets responsible for the utilization of a specific type of opine were evolved. *Agrobacterium* spp. appears to have undergone a quite peculiar evolutionary journey to survive in their interactions with plants. In the process, opines have played important roles. Close examination of divergent forms of genes involved in the metabolism of opine will provide us with valuable sources for the study of inter-kingdom relationships between microbes and hosts.

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